Mutations in the Adenomatous Polyposis Coli (APC) gene in patients with Familial Adenomatous Polyposis (FAP) with Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE)

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Abstract

Introduction

Familial Adenomatous Polyposis (FAP) is an autosomal dominant disease characterized by large numbers of adenomatous polyps in the colon. Ancillary features of FAP include congenital hypertrophy of the retinal pigment epithelium (CHRPE), desmoid tumours, osteomas, and epidermoid cysts. CHRPE is the most common extracolonic lesion of FAP and can be identified early after birth. It is regarded as a useful clinical marker for FAP. Aberrations in adenomatous polyposis coli (APC) gene are casual for FAP. More than 700 APC mutations have been reported in Caucasians and Japanese, but only a 5-base pair (AAAGA) deletion in exon 15 has been reported in Chinese. The purpose of this study is to identify APC mutations in Chinese FAP patients and to assess the presymptomatic diagnostic value of CHRPE in FAP.

Methodology

A total of 55 subjects were recruited including 10 FAP index patients, 33 family members from 6 families, 4 controls with CHRPE only (unassociated with FAP) and 8 healthy controls. Fifty of the 55 study subjects were examined by indirect ophthalmoscopy. The criterion for CHRPE was the presence of 3 or more CHRPE lesions in one or both eyes. DNA was extracted from venous blood in all the study subjects for detection of sequence alterations in exons 6-15 of the APC gene by Polymerase Chain Reaction (PCR), Single Strand Conformation Polymorphism (SSCP) and direct DNA sequencing. Haplotype analysis was performed using 5 microsatellite markers flanking the APC gene in 4 FAP families.

Results

Nine of the 10 FAP patients (the remaining one was not examined by ophthalmoscopy because he was terminally ill in FAP at the time of our study) had one or two CHRPE lesions (100%) and 7 had more than 3 lesions in both eyes (77.8%). Fundal examination was performed on 29 of the 33 family members. Eight of the 29 family members had one or two CHRPE lesions (27.6%), but only 2 had more than 3 (6.9%) lesions. CHRPE lesions were not detected in any of the 8 control subjects. The sensitivity and specificity of CHRPE in FAP were 77.8% and 94.6% respectively. In the APC gene, a novel single base insertion at codon 1023 (3068insA) leading to a stop codon was detected in one FAP patient. Three previously reported mutations were also found: a deletion of 5 base pairs (ACAAA) at codon 1061 (3183del5), two truncating single base substitutions at codon 216 (R216X) and 283 (R283X), all the patients with these mutations are heterozygous. We also found 3 new silent mutations (1050T/G, 4965A/G and G→A in intron 10) and 4 polymorphisms (1458C/T, 1635A/G, 4479G/A and 5034A/G). No APC mutation was found in 3 out of the 10 FAP patients. A high risk haplotype based in microsatellite markers flanking the APC gene was found in one FAP family.

Discussion

The incidence of CHRPE in FAP in this study was 77.8% which was within the range 66 to 97% as reported in Japanese and Caucasians. The sensitivity and specificity of CHRPE in our FAP patients, 0.78 and 0.95 respectively, were also similar to other reports. Although the number of FAP patients was small, such results were indicative of the possible use of CHRPE as a clinical marker for FAP as in other populations. The novel mutation 3068insA was found in a 29-year-old FAP patient who had 15 CHRPE lesions in both eyes. The previously reported 3183del5 mutation is a hot spot in the APC gene. It was detected in a 36-year-old FAP woman with 18

CHRPE lesions in both eyes. The R216X mutation was found in one FAP patient who showed 5 CHRPE lesions in both eyes. The R283X mutation was detected in 4 FAP patients and 1 non-FAP member in one family, 3 of these 5 subjects had more than 3 CHRPE lesions. Our study indicated that CHRPE lesions perhaps are serious if mutations occur at codon 1023 and 1061, and CHRPE lesions are present if mutation occurs before exon 9 of the APC gene. But further analysis of a large number of patients with mutations is required to confirm whether the position of the APC gene mutation is correlated with CHRPE. Although a high risk haplotype was determined in one FAP family using microsatellite markers flanking the APC gene, but the haplotype analysis was still limited due to small family members were studied.

Conclusions

The novel and all 3 previously reported APC mutations found in this study segregated with CHRPE in FAP patients. The site of an APC mutation influence CHRPE expression, but is not the only factor responsible for the presence and number of retinal lesion in FAP patients. CHRPE itself shows sufficient sensitivity and specificity as a clinical marker for FAP. While opthalmologic examination is a direct, noninvasive and inexpensive test for FAP screening, occurrence of APC mutations in asymptomatic individuals is also indicative of endoscopy to look for FAP.

摘要

导言

家族性腺瘤性息肉病(FAP)是一种常染色体显性遗传病，表现为结肠大量腺瘤性息肉。FAP的其他体征还包括：先天性视网膜色素上皮增生(CHRPE)，纤维样瘤，骨肿瘤，以及表皮样囊肿。CHRPE是FAP最常见的肠外病灶，且早在出生后即可检查到。故CHRPE被看作是FAP的一个有用的临床标记。FAP是由腺瘤性结肠息肉基因(APC)突变导致的。在白种人群和日本人群中已报导的APC基因突变有700余种，但是在中国人群中仅报导一例在第15外显子中有一个5碱基对(AAAGA)缺失。本研究的目的是在中国人群的FAP病人中检测APC基因的突变，并评估CHRPE做为前驱症状诊断FAP的价值。

方法

研究对象共有55例，其中10例确诊为FAP，33例是分别来自6个FAP家族的家庭成员，4例仅患CHRPE做为对照，8例是健康对照。55例中有50例经间接检眼镜检查。CHRPE的体征是单侧或双侧眼底具有3个或以上的色素上皮细胞增生病灶。我们从所有研究对象的静脉血中提取DNA做PCR，SSCP及直接DNA测序以检测APC基因第6至15外显子的DNA序列变化。用5个microsatellite标记物分别侧接4个FAP家族的APC基因进行单倍型分析(haplotype analysis)。

结果

10例FAP病人中有9例(另1例由于在我们研究过程中因FAP过世而没有经检眼镜检查)具有1个或2个CHRPE病灶(占100%)，且其中7例双眼均有3个以上病灶(占77.8%)。在33个家庭成员中有29人经眼底检查，其中有8例具有1或2个CHRPE病灶(27.6%)，但仅有2例具有3个以上病灶(6.9%)。在8例正常对照中未查到CHRPE。CHRPE对FAP的灵敏度和特异性分别为77.8%和94.6%。在1例FAP患者中，检测到一个新的单碱基插入，位于APC基因的第1023个遗传密码(3068insA)，使之成为终止码。我们也发现3个曾经报导过的突变点：一个5碱基对(ACAAA)的缺失位于第1061密码(3183del5)，2个在第216(R216X)和第283(R283X)密码的截短单碱基替代，所有上述均为异源型。我们也发现3个新的静止突变(在第10内显子中，1050T/G，4965A/G及G→A)，以及4个多态现象(1458C/T，1635A/G，4479G/A，及5034A/G)。在10例FAP病人中有3例无 APC基因突变。在一个FAP家族中用标记物侧接APC基因发现一例高危单倍型。

讨论

本研究中，FAP病人的CHRPE发病率为77.8%，其范围符合日本人及白种人群的66%至97%的报导。CHRPE对我们的FAP病人的敏感度和特异性分别为0.78和0.95，与其他报导无异。尽管FAP病例较少，但此结果仍可提示CHRP可在其他人群中作为FAP的临床指标。我们在一位29岁的FAP病人APC基因中发现了新的突变(3068insA)，其双眼共有15个CHRPE病灶。以往报导指出APC基因突变大多数发生于3183del5。在我们一位36岁FAP女病人中也发现此突变，其CHRPE病灶双眼共18个。我们在一位有5个CHRPE病灶之病人APC基因中检测到R216X突变。R283X突变则发现于一个FAP家族中的4位FAP病人和1位非FAP成员，其中3位病人具有3个以上CHRPE病灶。我们的研究显示如果突变位于第1023和1061密码，则CHRPE表现很严重，且位于APC基因第9外显子之前的突变均患有CHRPE。但是APC基因突变的位置是否与CHRPE相关有待进一步的大量病例分析。尽管用microsatellite标记物侧接APC基因的方法查到一个FAP家庭中的高危单倍型，但是此分析仍因为样本量较少而有局限性。

结论

我们在患有CHRPE的FAP病人中发现了1个新的和3个曾报导过的APC基因突变。APC基因突变的位置影响CHRPE的表达。但这并非决定FAP病人视网膜病灶的发生与数量的唯一因素。CHRPE本身作为FAP临床指标具有一定的敏感度和特异性。而作为FAP筛选普查，眼科检查具有直接而非介入性及费用低廉等优点，然而具有APC突变之无症状个体仍应接受内窥镜检查以确诊FAP。

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Abbreviations

Amino acids:

A	Asn	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asp	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
X		stop codon

Nucleotides:

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

General:

APC	Adenomatous Polyposis Coli
bp	Base pair
cDNA	Complementary DNA
CHRPE	Congenital Hypertrophy of the Retinal Pigment Epithelium
Del	Deletion
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotides
ddNTP	Dideoxynucleotides
EDTA	Ethylenediamine-tetraacetate di-sodium salt
FAP	Familial Adenomatous Polyposis
Ins	Insertion
kb	Kilobase
mRNA	Messenger ribonucleic acid
PCR	Polymerase Chain Reaction
RFLP	Restriction fragment length polymorphisms
RNA	Ribonucleic acid
SSCP	Single strand conformation polymorphism
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TEMED	N,N,N',N',-tetramethyl ethylenediamine
UV	Ultra violet

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Chapter 1 Introduction

1.1 Familial Adenomatous Polyposis (FAP)

Familial Adenomatous Polyposis (FAP) is a dominant inherited disease characterized by the development of hundreds to thousands of adenomatous polyps in the colon (Utsunomiya et al. 1990). The disease is caused by mutations in the tumor suppressor gene APC (Adenomatous Polyposis Coli) on chromosome 5q21-22 (Groden et al., 1991; Joslyn et al., 1991; Kinzler et al., 1991 and Nishisho et al., 1991).

1.1.1 Occurrence and prevalence

FAP has been described for more than a century and was first reported by Sklifosovske in 1881. Smith found the association of FAP with cancer in 1887. In 1890, Bickersteth discovered its genetic origin, and subsequently Cockayne discovered an autosomal dominant mode of inheritance in 1927. The account of FAP is about 1% in all colorectal cancers (Burt et al., 1985). Epidemiologic data suggest that the incidence of FAP range from 1:8,000 to 1:15,000 after birth (Bulow et al., 1986; Utsunomiya et al., 1990 and Jarvinen et al., 1992). The estimation of FAP frequency is mainly based on indirect calculations from restricted regional samples. The proportion of isolated cases of FAP ranges from 22% to 46% in registry-based studies (Maher et al., 1993; Bisgaard et al., 1994).

1.1.2 Clinical features

Colonic manifestations

Familial adenomatous polyposis (FAP) is a common and highly penetrant genetic disease. It is characterized by numerous adenomatous polyps (>100) in the colon and rectum (Murday & Slack, 1989) (Figure 1-1). In some extreme cases the bowel is carpeted with a myriad of polyps. The affected individual typically develops multiple polyps in the colon during the second or third decade of life. One or more of these adenomas will inevitably progress to adenocarcinoma unless the affected colon is removed (Bussey et al., 1990). The presenting features are usually those of malignancy such as weight loss, inanition, bowel obstruction or bloody diarrhea. About one third of the cases have no family history; the isolated cases that present with symptoms tend to be diagnosed at a later age, often with moderate to severe dysplasia or even adenocarcinoma (Powell et al., 1990).

Extracolonic manifestations

Majority of FAP patients has one or more extracolonic features (Krush et al., 1988). Gardner's syndrome and FAP both occur in siblings, and have been mapped to the same chromosomal location (Bodmer et al., 1987; Nakamura et al., 1988). They may be associated with identical pathological mutations in the APC gene (Nishisho et al., 1991), so the terms are regarded as synonymous. There is evidence to suggest that some extracolonic features of the disease correlate with the nature of the mutations. Extracolonic features in FAP can be classified into 3 groups:

(1) Adenomatous polyps in the upper gastrointestinal tract

This extracolonic feature has become a major determinant of long-term morbidity. Periampullary cancer is a well-recognized feature of FAP (Jones et al., 1977; Harned et al., 1982). Offerhaus et al. (1992) found that there was a greatly increased risk of duodenal adenocarcinoma and ampullary adenocarcinoma in FAP patients. No significant increased risk was found for gastric or nonduodenal small intestinal cancer. Burt et al. (1984) suggested that the prevalence of gastric cancer in Japan may be associated with the greater frequency of this type of malignancy in Japanese carriers of the APC gene defect.

(2) Ocular, cutaneous, and skeletal features

Blair et al. (1980) pointed out that congenital hypertrophy of the retinal pigment epithelium (CHRPE) is an usual feature present in Gardner's syndrome and can be a valuable clue to the predisposition of Gardner's syndrome in persons who have not yet developed other manifestations. Consequently, CHRPE is recognized as the most common extracolonic manifestation of FAP and an early clinical marker of the disease (Diaz-Llopis et al., 1988; Lyons et al., 1988). CHRPE would be reviewed in this section later. In Gardner syndrome, polyps of the colon and sometimes of the stomach and small intestine are associated with osseous and soft tissue tumors. Globoid osteomata of the mandible with overlying fibromata are characteristic (Ushio et al., 1976; Jagelman et al., 1987). Osteomatous changes in the calvaria with associated fibromas (of the forehead, for example) are also noted. Sebaceous or epidermoid cysts occur on the back. Dental anomalies in Gardner's syndrome were first described by Fader et al. (1962).

Jarvinen et al. (1982) found jaw osteomata in 76% and dental anomalies in 18% of patients. These dental anomalies include impacted teeth, supernumerary teeth, congenitally missing teeth, and abnormally long and pointed roots on the posterior teeth (Carl et al., 1987).

(3) Tumors and malignancy in organs outside the gastrointestinal tract

FAP registry studies have shown that occurrence of tumors and malignancy is not limited to the gastrointestinal tract of patients with FAP. Some patients are affected with desmoid tumors, hepatoblastoma, thyroid carcinoma, mesenteric fibromatosis and other rare forms of cancer or neoplasm. The slow growing lesions of desmoid tumors are invasive and can reach enormous sizes. Though usually located with the abdominal cavity, they may develop anywhere in the body. Some studies have identified desmoid tumors (from 6% to 31%) to be one of the most common extracolonic causes of mortality (Arvanitis et al., 1990; Bertario et al., 1994; Belchetz et al., 1996). Klemmer et al. (1987) found an increase in the frequency of desmoids in persons with FAP of the colon, but the risk was dependent on age and sex. The lifetime risk was estimated to be 8% for males and 13% for females. It affected more elderly than young people. The incidence of hepatoblastoma in FAP patients is 1 in 100,000 (Giardiello et al., 1991). Thyroid carcinoma in Gardner's syndrome was first described by Camiel et al. (1968). In 1995, Herve et al. reviewed a large number of published references and estimated that the incidence of thyroid carcinoma in patients with Gardner's syndrome approached 100 times that of the general population. Some patients may develop fibromatosis, especially after surgery (Simpson et al., 1964).

1.1.3 Laboratory studies

Histological findings

FAP is characterized by multiple adenomas, averaging less than 5 mm in diameter; the often cited number of 100 varies according to the age of the patient. There is no histologic difference between adenomas in FAP and those in the general population. Morson and Bussey (1970) identified a progressive sequence of epithelial dysplasia with adenomatous tissue at the margins of intestinal cancers, establishing the adenoma as a cancer precursor.

Biochemistry findings

The activity of ornithine decarboxylase may be a useful marker for the genotype of familial polyposis (Luk et al., 1984). This rate-limiting enzyme in the polyamine biosynthetic pathway is essential for intestinal mucosal proliferation. Mucosa from dysplastic polyps showed elevated ornithine decarboxylase activity when compared to non-dysplastic polyps. Smith et al. (1993) produced monoclonal and polyclonal antibodies to adenomatous polyposis coli to characterize the protein in normal and tumor cells. They found that 81% of colon tumor cell lines were totally devoid of the normal, full-length protein, whereas 40 cell lines derived from sporadic tumors of other organs had full-length APC. Immunohistochemical analysis of APC in normal colonic mucosa demonstrated cytoplasmic staining, with higher intensity staining in the basolateral margins of the epithelial cells. The staining was markedly increased in the upper portions of the crypts, suggesting an increased level of expression with maturation.

Other findings

Skin fibroblasts from patients with Gardner's syndrome and familial polyposis coli were reported to show increased susceptibility to retrovirus-induced transformation and chromosomal aneuploidy (Rasheed et al., 1983). Chen et al. (1989) demonstrated that the in vitro life span of cultured skin fibroblasts from individuals with FAP was markedly extended when compared with that of normal individuals. Midgley et al. (1997) developed antisera to APC N- and C-terminal epitopes, and found that the APC protein was expressed in epithelial and mesenchymal cells in many tissues. In the epithelium of bladder, small and large intestine, esophagus, stomach, and epidermis, APC expression was restricted to regions where cell replication has ceased and terminal differentiation is established. The authors concluded that this distribution is compatible with APC function related to signaling at the adherens junction and indicates that APC plays a role in cells committed to terminal differentiation.

1.1.4 Diagnosis

Endoscopy

It is very important to examine the affected family members and at-risk adolescents and siblings, because the affected individual with hundreds of polyps may remain clinically asymptomatic. When clinical symptoms are apparent, about two-thirds of such patients would have already developed carcinoma of the colon or rectum (Powell et al., 1990). Consequently, screening guidelines have been established for ages 10 to 14 years. Frequent examination of at risk subjects

by sigmoidoscopy beginning in the early teens is recommended to detect early adenomas. Flexible sigmoidoscopy remains the standard screening procedure because it is minimally invasive, allows for biopsy, and is generally acceptable to adolescents. The use of barium enema is discouraged because of the propensity to overlook early-stage adenomas of size around 1 to 2-mm. It is recommended every 2 years until age 35 and every 3 to 5 years thereafter for at-risk first-degree relatives who have not undergone predictive testing or who are uninformative subsequent to DNA analysis. Because the offspring of a patient with FAP has a 50% chance of inheriting the defective gene and puberty is the usual age of onset, an adolescent with less than 10 adenomas and a confirmed family history of FAP should be considered as affected.

Dental Panoramic Radiographs Score (DPRS)

A weighted scoring system for changes on dental panoramic radiographs called the Dental Panoramic Radiographs Score (DPRS) could be used as a diagnostic tool for FAP (Thakker et al., 1995). The score took into account the nature, extent, and sight of osseous and dental changes, as well as the incidence of the anomaly in the general population. Using the highest threshold, a specificity of 100% and sensitivity of approximately 68% were obtained. If all positive findings were considered as significant, sensitivity was increased to approximately 82%, but the specificity was reduced to approximately 88%. Overall, approximately 68% of the affected subjects had significant changes, and approximately 18% had normal appearance on DPR, with the remainder having changes classified as minimal or equivocal.

1.1.4 Management

Waddell and Loughry (1983) were the first to make a connection between nonsteroidal anti-inflammatory drugs (NSAIDs) and colon cancer. They observed the disappearance of rectal polyps in a patient with Gardner's syndrome and correctly attributed this disappearance to treatment with sulindac, an NSAID that was given for unrelated reasons. Giardiello et al. (1993) found that sulindac, at a dose of 150 mg orally twice a day for 9 months, reduced the number and size of colorectal adenomas. The effect has been confirmed in numerous clinical trials (Marcus et al., 1995) and the interpretation that NSAIDs promote regression of colon polyps by inhibiting prostaglandin synthesis has been supported by genetic studies in mice (Oshima et al., 1996). However, up to now, prophylactic colectomy is the preferred and possibly the only effective management for those who have developed polyps and are positive gene carriers.

1.2 Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE)

Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE) was first discribed by Reese and Jones in 1956 in a subject free of other diseases. CHRPE is a benign fundus lesion and is usually discovered during routine ophthalmoscopy. But Blair and Trempe (1980) pointed out that CHRPE is a frequent finding in Gardner syndrome and can be a valuable clue to the presence of the gene in persons who have not yet developed other manifestations.

1.2.1 Clinical features

Ophthalmoscopic features

CHRPE independent of FAP

A typical fundus lesion of CHRPE shows a unilateral, solitary, round, flat, circumscribed and hyperpigmented lesion. It is well demarcated from normal-appearing retinal pigment epithelium (RPE) and is not known to be associated with other ocular or systemic disorders. It may show chorioretinal atrophy at the center of the lesion encircled by a narrow halo of hypopigmented RPE. The color of the lesion can range from light grayish-brown to black, independent of the race of the person (Kurz et al., 1962). Punched-out hypopigmented or depigmented lacunae may be present within larger lesions. Clinically, the overlying retina and its vasculature appears normal in the majority of cases. Some cases showed a longitudinal strip of whitish change following the course of an artery, appeared to enclose the blood vessel like a sheath, or a longitudinally orientated area of hypo- and hyperpigmentation close to the retinal veins, or hyperpigmentation of the RPE under retinal vessels (Schmidt et al., 1994). Occasionally, there may be capillary and large-vessel obliteration and neovascularization (Cleary et al., 1976). The lesion varies in size from less than 100μm to several disk diameters and can be found anywhere in the fundus. While growth of CHRPE is seldomly observed (Norris et al., 1976), depigmented lacunae frequently enlarge or new lacunae may develop within the lesion (Buettner et al., 1975). When several lesions of varying sizes are arranged in a cluster, resembling the footprint of an animal, they are also referred to as grouped pigmentations or "bear tracks". Figure 1-2 shows a picture of CHRPE which is not associated with FAP.

CHRPE in FAP

Lewis et al. (1984) described multiple and bilateral patches of CHRPE in affected members of 3 families with Gardner's syndrome. The lesions of CHRPE in FAP are multiple, bilateral and smaller than lesions unassociated with FAP; some are as small as 50 to 100 μm in diameter (Figure 1-3). The larger lesions may be surrounded by a depigmented halo, contain depigmented lacunae, or may be accompanied by small satellite lesions. The appearance of CHRPE lesions is classified into 5 types (Berk et al., 1988), but the oval pigmented lesion with a pale halo is unique to FAP (Polkinghorne et al., 1990). The type, size and location of CHRPE in FAP are summarized in Table 1-1. Small pigmented black dots in the periphery of the retina, the most common lesion in FAP, also occur in normal subjects, but in these normal subjects more than three lesions are rarely found. Table 1-2 lists the differences between CHRPE in FAP and CHRPE unassociated with FAP.

Vision and visual field

CHRPE in FAP is asymptomatic and the benign pigmented fundus lesion is the same as that of non-FAP. Vision and visual field examination usually is normal, and patients generally do not complain of any symptoms related to the lesion, except when the lesion involves the macula. Detailed examination finds the lesions to be associated with corresponding scotomas in the visual field. These scotomas tend to be relative in younger patients, but appear absolute in older patients. The depth of the scotoma increases with the age of the patient, but these lesions do not grow in size (Buettner et al., 1975).

Fluorescein angiographic features

The RPE and choroid adjacent to the area of CHRPE show a normal fluorescein angiographic pattern (Buettner et al., 1975). The hypertrophic RPE blocks background choroidal fluorescence, augmenting the bright contrast of the retinal vasculature. Through depigmented lacunae or hypopigmented halo, a normal choriocapillaris flush is observed. Although leakage of fluorescein dye from retinal vessels overlying CHRPE has been reported (Cleary et al., 1976), in the majority of lesions no leakage of dye is observed during any phase of the angiogram.

Electrophysiologic findings

The electro-oculogram (EOG) and the electroretinogram (ERG) show normal patterns in eyes with CHRPE (Santos et al., 1994).

1.2.2 Pathogenesis

CHRPE in FAP

It was suggested that CHRPE may be associated with APC gene mutations (Olschwang et al., 1993b; Wallis et al., 1994; Kartheuser et al., 1995; Caspari et al., 1995; Davies et al., 1995). These mutations lead to the synthesis of a truncated protein. The extension of CHRPE was found to be dependent on the length of the truncated protein, and the position of mutation along the coded sequence. Exon 9 of the APC gene is a critical boundary for the presence or near absence

of CHRPE. Retinal lesions are almost always absent if the mutation occurs before exon 9, but are consistently present if the mutation occurs after this exon.

CHRPE independent of FAP

Observation of CHRPE in newborn infants and young children provides evidence for the congenital nature of this lesion (Champion et al., 1989). Relative scotomas with mild photoreceptor degeneration in young patients compared to deep, absolute scotomas with advanced photoreceptor degeneration in old patients suggest that the abnormality is limited to the RPE. Presumably, the abnormal RPE can not maintain the integrity of the photoreceptor cells nearby, resulting in not only progressive degeneration totally but also RPE cell death and formation of depigmented lacunae. Ultraviolet fluorescence microscopy showed no autofluorescent granules of lipofuscin in the CHRPE, suggesting that the lesion's constituent cells lack the capacity for phagocytosis and digestion of photoreceptor outer segments (Lloyd et al. 1990). Other studies classified CHRPE lesions as hamartomas of the RPE (Kasner et al. 1992), or as melanotic nevus (Roseman et al. 1992).

1.2.3 Histology

Histopathology reveals a single layer of hypertrophic cells, containing numerous large pigment granules, resting on a markedly thickened and strongly positive acid-Schiff (PAS)-positive Bruch's membrane (Buettner et al., 1975; Lloyd et al., 1990). Hyperplastic changes in the form of multiple layers of hypertrophic RPE cells have also been reported (Wirz et al., 1982; Kasner et al., 1992). The choriocapillaris and the remainder of the choroid are normal underneath the

CHRPE lesion. The transition from normal to hypertrophied RPE occurs abruptly. Overlying the hypertrophied RPE, the retinal photoreceptor cell layer is markedly degenerated with only a few remaining inner and outer segments within amorphous, PAS-positive material. In younger patients the photoreceptor cells overlying CHRPE exhibit less advanced degeneration than in older people. The inner retinal layers, including the retinal vasculature, are normal. The hypopigmented halo, found to surround some lesions of CHRPE, consists of slightly less pigmented hypertrophied RPE cell. In the area of the depigmented lacunae, both the hypertrophic RPE and photoreceptor cell layer are absent and have been replaced by a multicellular layer of glial cells interposed between Bruch's membrane and the normal inner retinal layers (Buettner et al., 1975). Under electron microscopy, the thickened Bruch's membrane consists of marked widening of the basement membrane of the hypertrophic RPE cells. Surrounding the CHRPE lesion, the retina and pigment epithelium is normal. In the area of hypertrophy, the RPE cells are markedly thickened and densely packed with large round pigment granules instead of the wedgeshaped melanin granules found in normal RPE cells (Champion et al., 1989). The photoreceptor outer segments are missing overlying the abnormal RPE cells.

1.2.4 Differential diagnosis

In the vast majority of cases, pigmented fundus lesions are easily recognized when using the indirect ophthalmoscope and scleral indentation. However, in order to avoid missing out very small and lightly pigmented lesions, a three-mirror contact lens examination is necessary when no lesions are detected with indirect ophthalmoscopy. Because small numbers of CHRPE may occur in normal subjects, the threshold for diagnosing CHRPE positive in FAP are three or more

lesions in one or both eyes (Lewis et al., 1984; Romania et al., 1989). CHRPE can easily be identified correctly by modern examination techniques. However, descriptions of this lesion in earlier reports, along with reviews of older histopathologic data, suggest that CHRPE have been frequently misdiagnosed. Most often it has been mistaken for malignant melanoma of the choroid, choroidal melanomas, choroidal nevi, melanocytomas, hyperplasia of the RPE or focal pigmentation. Choroidal melanomas are almost always elevated, less uniformly pigmented or less well demarcated as CHRPE, and exhibit growth. Choroidal nevi are located deep to the RPE and are flat. Their color may vary from light to dark brown. The borders are less well demarcated than those of CHRPEs and are often feathery because nevus cells extend along larger choroidal vessels. Melanocytomas of the choroid have a similar appearance, except for their black color. True hyperplasia of the RPE also has ill-defined borders and invades the retina, often resulting in distortion of the retina. Focal pigmentation caused by injury, inflammation or drug toxicity may resemble CHRPE, but can be differentiated by its more irregular shape, widespread distribution, and associated clues suggesting its acquired nature.

1.2.5 CHRPE as an early clinical marker for FAP

Traboulsi (1987) reported that the specificity and sensitivity of bilateral multiple retinal lesions as a marker for FAP with extracolonic manifestation were 0.95 and 0.78 respectiviely. Diaz-Llopis et al. (1988) suggested that CHRPE may be a useful marker to detect patients at risk for FAP in families who carry the mutated gene. The combination of features obtained from eye examination for CHRPE, information on the age of onset and linked DNA markers appeared to be highly effective in carrier detection. Lyons et al. (1988) concluded that the CHRPE phenotype

is a more powerful marker than other phenotypic features of Gardner's syndrome. Ophthalmic examination was performed on 56 patients with 'validated' FAP, and 8 patients were found to have the retinal lesions without any of the extracolonic features of Gardner's syndrome. It is possible that the eye lesion may be the only extracolonic feature of Gardner's syndrome (Baker et al., 1988). Houlston et al. (1992) suggested that CHRPE is not an exclusive manifestation of mutation at the APC locus. Patients expressing CHRPE tend to cluster within specific polyposis families. The proportion of cases of FAP that manifest significant numbers of CHRPE varies from 66 to 92% in recent studies (66% by Baker et al., 1988 and Romania et al., 1989; 80% by Polkinghorne et al., 1990; 87.5% by Berk et al., 1988; 92% by Burn et al., 1991). The explanation for these variations is not clear, but it is possible that different ophthalmologists may vary in their criteria in recording retinal abnormalities, especially for small lesions. The patient populations may also differ. In Chinese, the incidence of CHRPE in FAP patients was 100% in a small study involving 7 FAP families and 11 index patients (Lam et al., 1998). A subject at risk of inheriting the gene for FAP and whose retinae contained three lesions was estimated to have a probability of 67% as a carrier, while the presence of more than three lesions was diagnostic (Burn et al., 1991). Since the colonic and extracolonic manifestations of FAP do not develop until the second or third decade of life, the discovery of CHRPE at an early age in a person at risk for FAP is a valuable aid for genetic counseling and prognostic management. Colectomy at the time when polyps develop, long before the progression to adenocarcinoma, is the lifesaving treatment of choice. However, the absence of CHRPE lesions in a person at risk does not indicate the absence of the gene, since variable ocular expressions of this marker gene exist as with all dominantly inherited traits. The detection of multiple, bilateral CHRPE in members of families

with inherited intestinal polyposis allows the ophthalmologist to make an important contribution to the early recognition of persons at risk for the development of intestinal malignancies.

1.3 The Adenomatous Polyposis Coli (APC) Gene

1.3.1 Discovery

The chromosomal location of the gene responsible for FAP has been unclear for a long time, because no specific deletion has been detected in chromosomes of either the normal or tumor tissues from FAP patients. In 1986, Herrea et al. first detected a constitutional interstitial deletion of 5q in lymphoid cells from a patient with Gardner's syndrome. In 1987, the disease gene was mapped to chromosome 5q21-22 by linkage analysis using restriction fragment length polymorphisms (RFLPs) (Bodmer et al. and Leppert et al.). Loss of heterozygosity (LOH) on chromosome 5q was detected in sporadic colorectal carcinomas in 1987 (Solomon et al.), and later in FAP carcinomas by Okamoto et al. in 1988 using RFLP probe C11p11. Other RFLP probes, including YN5.48, LS5.34, and L5.71 have since been isolated (Meera et al., 1988; Nakamura et al., 1988; Dunlop et al., 1990) and confirmed to be closer to the disease gene by linkage analysis in FAP families. LOH on chromosome 5 suggested that the disease gene is the mutant of a tumor suppressor gene. In 1991, isolation of the APC gene was achieved by four groups (Groden et al., Joslyn et al., Kinzler et al. and Nishisho et al.) using RFLP probes close to the APC gene as markers. Consequently, it became possible to clarify the molecular mechanisms of tumor formation in FAP patients.

1.3.2 Structure and function

The APC gene is located on chromosome 5q21-22 and consists of 15 exons and 14 introns spanning more than 100 kilobases (Kinzler et al., 1991) and encoding 2843 amino acids (Figure 1-4). Exons 1-14 vary in size from 78 (exon 12) to 379 bp (exon 9), accounting for 23% of the protein coding regions of the gene. Exon 15, however, is remarkable for its size, comprising 77% of the protein coding sequence. It is one of the largest eukaryotic exons ever described. The predicted APC protein is hydrophilic, and it has no potential signal peptides corresponding to transmembranal regions, or nuclear targeting signals, suggesting a cytoplasmic localization of the protein. The peptide structure has local sequences homologous to myosin and keratin. In the NH_2-terminal and the central portion, the gene contains sequences that have coiled-coil potential, which indicates that the APC protein has the ability to form homo- and hetero-oligomers.

The function of APC remains unclear but its cellular localization and interactions with other protein led to several hypothesis. Rubinfeld et al. and Su et al. (1993a) found that the APC protein is associated with adherens junction protein alpha- and beta-catenin. These proteins bind to the cell surface molecule, E-cadherin, and are essential in cellular adhesion. The APC protein may affect the interaction between catenins and E-cadherin, thus influencing cellular adhesion and possibly intercellular communications. Antibodies to the APC protein have identified a 300-kDa cytoplasmic protein expressed in epithelial cells in the upper portions of the colonic crypts, suggesting that it is functional in the mature colonocytes (Smith et al., 1993). Subcellular localization has demonstrated that wild-type APC is closely associated with microtubule formation. However, mutant APC proteins do not bind to microtubules (Munemitsu et al., 1994; Smith et al., 1994). In vitro studies indicate that wild-type APC not only binds to microtubules but also promotes their formation (Munemitsu et al., 1994). Since the organization and structure

of microtubules are vital to cell division, it is possible that the association of APC with the catenins, and hence cadherin, may afford a direct line of intracellular communication between the cell surface and the site of microtubule formation. Mutant APC protein has been shown to interact with normal APC, forming a heterodimer which may result in defective function in a dominant-negative manner (Su et al., 1993b). The extent to which normal APC function is impaired may be related to the structure and residual ability of the mutant protein to form dimers. Truncated APC protein due to mutations occurring in the most 5′ part of the gene may not be capable of dimerization with wild-type APC whereas the more 3′ mutations may allow translation of repeat sequences which can allow dimer formation. Thus, it might be predicted that a more aggressive phenotype may result from the more 3′ mutations.

1.3.3 Sequence alterations in the APC gene

Since the coding sequence of the APC gene has been identified, examination of its mutations became possible. The coding sequence includes 8972 nucleotides and the search for mutations is rather laborious, but analysis of mutations of the APC gene is important for direct presymptomatic diagnosis of patients not only in FAP families, but also in those without family history. Up to now, over 700 both germline and somatic mutations have been reported (Christophe et al., 1996). More than 98% of the mutations are frameshift or nonsense mutations leading to the synthesis of a truncated protein. This feature clearly indicates that each of these mutations is truly deleterious for APC function. Common mutations of the APC gene are summaried in Table 1-3.

Germ-line mutations of the APC gene in FAP patients

Mor than 300 germline mutations of the APC gene have been reported in unrelated FAP patients (Christophe et al., 1996). Miyoshi et al. (1992a) detected 53 germ-line mutations in the APC gene in 79 unrelated FAP patients using ribonuclease protection analysis coupled with PCR amplification of genomic DNA. More than 90% of these mutations appeared to result in truncation of the APC protein. Nearly 70% of the mutations were clustered in the 5′ half of exon 15, and nearly 20% of the total mutations occurred at 5 positions: codons 1309, 1061, 1546, 625 and 302. Germline mutations scattered throughout the 5′ half of the gene, but two hot spot codons have been identified, one at codon 1061 including the AAAAC 5-base-pair or AAAC 4-base-pair deletion and the other at codon 1309 including the AAAAG 5-base-pair deletion (Miyoshi et al., 1992a; Cottrell et al., 1992; Groden et al., 1993; Varesco et al; 1993, Miyaki et al., 1994). It is well known that deletions and insertions in the APC gene often occur at positions containing repeated sequences within the coding sequence, and all caused frameshifts, which resulted in stop codons downstream. One of the possible causes of such deletions and insertions is replication misalignment, which is liable to occur within homopolymeric sequences (Kunkel et al., 1990). For example, a 5-base (AAAGA) deletion from ATAAAAGAAAAGATT at codon 1309 and a 5-base (ACAAA) deletion from ATAAAACAAAGT at codon 1061 have been detected. It should also be noted that the APC protein truncated at these positions may be significant to the inactivation of the APC gene and the cause of the clinical phenotype in FAP patients. Other deletions or insertions also occurred in the region within or near the repeated sequence. Another characteristic of APC germline mutations is that more than 80% of the known base substitutions are from cytosine to other nucleotides. Such a high frequency of mutation at C may also be explained by the hypothesis that a large number of mutations from C and G occur as

a result of transient misalignment of the bases at the replication fork (Cooper et al., 1990). It may also be due to the fact that FAP is usually caused by inactivation of the APC gene by formation of stop codons TGA, TAG and TAA.

Somatic mutations of the APC gene in colorectal tumors from FAP and non-FAP patients

Extensive screening for somatic mutations of the APC gene has been carried out in colorectal tumors. More than 400 APC somatic mutations of the APC gene have been described (Christophe et al., 1996). A Japanese group screened 138 colorectal adenomas and carcinomas including 87 from FAP patients, and found 65 somatic mutations (Miki et al., 1992; Miyoshi et al., 1992b; Ichii et al., 1992, 1993). Powell et al. performed direct DNA sequencing of the entire coding region of this gene and detected 35 somatic mutations in 41 colorectal tumors (1992). Miyoshi et al. (1992b) reported that over 60% of the somatic mutations in the APC gene were clustered within a small region of exon 15, called the mutation cluster region (MCR). Approximately 94% of the FAP somatic mutations were clustered between codon 1280 and 1500. In contrast, only 29% of germ-line mutations occurred in this region. In 1995, Michiko et al. reported several hot spots of somatic mutations in the APC gene including codons 1307-1311, 1378, 1450, 1462-1465 and 1478-1490. Two of them, at codons 1307-1311 and 1462-1465, are hot spots in both germ-line and somatic mutations, but the other three positions were mutated only somatically at high frequency. Over 80% of colorectal tumors had at least one mutation in the APC gene (Miyoshi et al., 1992b). More than 60% of these had two genetic changes, mutation plus mutation, or mutation plus LOH. They concluded that two genetic alterations of the APC gene were necessary for tumor formation. Powell et al. (1992) also examined mutations of the APC gene in 41 non-FAP colorectal tumors. About 60% of colon cancer had at least one

mutation in the APC gene and 64% of these mutations had two genetic changes. It was suggested that two genetic changes in the APC gene give a great growth advantage, but may not be required for tumorigenesis to occur. Powell et al. also concluded that somatic mutations of the APC gene are associated with development of colorectal tumors, not only in FAP patients, but also in non-FAP sporadic cases.

Polymorphisms of the APC gene

More than 25 polymorphisms have been identified in the APC gene (Groden et al., 1991; Nagase et al., 1992a; Miyoshi et al., 1992; Powell et al., 1992; Nagase et al., 1993; Olschwang et al., 1993a) and they are summarized in Table 1-4. The allelic frequencies of the 5 most common polymorphisms were 0.43/0.57 (with or without a cutting site) at codon 486, 0.35/0.65 at codon 545, 0.35/0.65 at codon 1493, 0.64/0.36 at codon 1678 and 0.63/0.37 at codon 1960. Base substitution at codons 545 and 1960 do not affect the recognition site of any restriction enzyme but can be easily distinguished by the allele-specific PCR method (Prezan et al., 1992). Allelic frequencies among the other 20 polymorphisms were less than 0.05. This information should be useful for presymptomatic diagnosis of FAP as well as for studies to examine tumors for the loss of heterozygosity at the APC locus (Yusuke Nakamura 1994).

1.3.4 APC Mutations Associated with Specific Clinical Features

Some interesting features were observed by the analysis of APC mutations regarding the possible relationship between the localization of the mutation and specific phenotypes associated with the

disease. The specific phenotypes include profuse (more than 5000 adenomatous polyps in the colon) and sparse (the number of polyps is between 1000 and 2000) types in the number of colorectal polyps (Utsunomiya et al., 1990) and differences in the frequency of extracolonic manifestations (Jagelman et al., 1987). The correlation between the location of the mutation and the number of polyps in FAP patients has been noted by Nagase et al. (1992b). In the profuse type of FAP patients, the mutations (frameshift mainly) are concentrated between codon 1255 and 1467. In the sparse type of FAP patients, APC mutations (missense and frameshift) are located near the 5′ end (codon 97-157) of the APC gene (Spirio et al., 1992). It seems that the presence of germline mutations in the extreme 5′ of the APC coding region before codon 157 are correlated with an attenuated phenotype which has fewer polyps and delayed onset of the cancer (Spirio et al., 1993). Olschwang et al. (1993a) reported that the severity of Congenital Hypertrophy of the Retinal Pigment Epithelium (CHRPE) also depends on the position of the mutation. Retinal lesions are almost always absent if the mutation occurs before exon 9, but are consistently present if the mutation occurs after this exon. Exon 9 is a transitional exon, with CHRPE lesions being present if a mutation occurs in the 3′ half of the exon, and absent if the mutation occurs in the 5′ half of the exon. Further analysis of a large number of patients is required to determine whether a particular mutation is correlated with a specific phenotype.

1.3.5 Mutations of the APC gene in Chinese

There is only one report in the literature regarding mutations of the APC gene in FAP patients in the Chinese (Gan et al., 1994). Fifty-three FAP patients were from 7 families in HangZhou located in the southeastern region of China. Restriction Fragment Length Polymorphism (RFLP)

was performed using a specific restriction endonuclease to screen the hot spot mutation at codon 1309 of the APC gene in these 53 FAP patients. A deletion of 5-base pairs (AAAGA) at codon 1309-1311 was detected in 10 of 53 FAP patients. The results also agree with the examinations by endoscopy. They concluded that mutation patterns of the APC gene in Chinese FAP patients might be similar to those of Caucasians.

1.3.6 Methods for detecting mutations in the APC gene

There are many different techniques to detect mutations of the APC gene. Table 1-5 summarizes the advantages and disadvantages of the methods widely used in different laboratories.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (PCR) is used to make large a number of copies of a specific DNA sequence. A preparation of DNA is denatured. The single stranded preparation is annealed with two short oligonucleotide primer sequences that are complementary to sites on the opposite strands on either side of the target region. DNA polymerase is used to synthesize a single strand from the 3′-OH end of each primer. The entire cycle can then be repeated by denaturing the preparation and starting again (Mullis et al., 1986). For detecting the mutations of the APC gene in FAP patients, PCR is the basis for preparation of a target DNA fragment for the detection of base changes with different methods.

Single Strand Conformation Polymorhism (SSCP)

Single strand conformation polymorphism (SSCP) analysis is a simple method for detecting most sequence variations in a single strand of DNA. The conformation of a single strand DNA under nondenaturing conditions is uniquel depending upon its sequence composition. This conformation is usually different even if a single base has been changed. The difference in conformation can be detected as mobility shifts in non-denaturing gel electrophoresis (Orita et al., 1988). SSCP is performed by denaturing PCR products into single stranded form, cooling rapidly to allow single strand self-folding, separating in a non-denaturing acrylamide gel under constant electrophoretic conditions and detected by autoradiography or silver staining. The sensitivity of SSCP for detecting point mutations can be improved by optimizing the variables involved or using a variety of SSCP conditions. Factors that affect SSCP migration and detection include the length of the PCR fragment, the temperature of the gel during electrophoresis, the method of PCR denaturation, and the characteristics of the gel. The optimal length of a single strand is typically between 100-300 nucleotides (Hayashi et al., 1991). Lengthy PCR products may be restricted with endonucleases. SSCP is applied widely to screen for APC gene mutations in FAP patients and their family members (Miyoshi et al., 1992, Cottrell et al., 1992; Groden et al., 1993; Olschwang et al., 1993).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE is based on the principle that different DNA duplexes will show differences in migration as they are forced to migrate through an electrophoretic gel in which there is a gradient of increasing amounts of a denaturant, usually a chemical denaturant. Temperature gradient gel

electrophoresis is also sometimes used (Cariello et al., 1993). The DNA duplexes continue to migrate until they reach a position in the gel where the strands melt and separate, after which the denatured DNA does not migrate much further. A single base pair difference between a normal and a mutant DNA duplex is sufficient to cause them to migrate differently. DGGE is potentially highly sensitive and avoids radioisotopes. However, primers must be designed very carefully because the sequence amplified needs the right profile of melting domains. DGGE has been used to identify mutations of the APC gene (Riccardo et al., 1992).

Chemical Cleavage Mismatch (CCM)

The method is based on the detection of chemical reactions to cleave one strand of the DNA at the site of the mismatch. The test sample is mixed with labeled wild-type sequence, denatured and allowed to renature. If the test sequence differs from the wild type, heteroduplexes are formed which can be cleaved by chemical or enzymic treatment. Cleavage is revealed by observing short labeled fragments in a gel. CCM is one of the most sensitive methods of mutation detection. It can be applied to kilobase-length samples. The disadvantages are that the chemicals are very toxic and some practice is required before it works well. CCM was also applied to detect mutations of the APC gene (Smith-Ravin et al., 1994).

Heteroduplex Analysis (HAD)

Heteroduplexes form when PCR products from a heterozygous person are denatured then cooled to allow single mutant strands to base pair with complementary strands from the wild-type allele. Heteroduplexes in polyacrylamide gels can be detected as extra slow moving bands. Mutations

including insertions, deletions and most single base substitutions are detectable if the fragments under investigation are less than 200 base pairs (Keen et al., 1991). HAD is mostly used in combination with single-strand conformation analysis on a single gel. HAD was applied widely in screening the APC gene for mutations (Johanna et al., 1995; Dagan et al., 1996).

Restriction Fragment Length Polymorphisms (RFLP)

RFLPs are simply variations in the lengths of the DNA fragments produced by cleaving DNA molecules with specific restriction endonucleases. These RFLPs can be visualized directly when the fragments in DNA digests are separated by agarose gel electrophoresis, stained with ethidium bromide, and viewed under ultraviolet illumination. Individual RFLPs can be detected by using specific cDNA or genomic clones as radioactive hybridization probes on genomic Southern blots. Mutations alter a restriction site or are comprised of significantly large insertions or deletions will create the restriction enzyme cutting site and hence can be detected using this method. RFLP was used to detect known mutations of the APC gene in FAP and colorectal cancers (Nishisho et al., 1991).

Restriction Enzyme Analysis

The method is used to detect mutations that create or destroy the cutting site of a restriction enzyme. PCR products can be digested with the appropriate restriction enzyme, followed by gel electrophoresis. This method can only be applied to detect known mutations, and not all base substitutions cause a change at restriction enzyme cutting sites, so the method has limited use for

detecting all mutations. Some known mutations and polymorphisms of the APC gene have been identified using this method (Gloria et al., 1993; Shozo et al., 1994; Delhanty et al., 1995).

Direct DNA Sequencing

The method can detect all known and unknown mutations. Dideoxy chain termination is the most widely used approach (Sanger et al., 1997). This method relies on the ability of dideoxynucleotides, which lack the 3′ hydroxyl group critical to phosphoribosyl chain extension, to terminate a strand synthesis from a specific oligonucleotide priming site by a DNA polymerase. The template must be single-stranded DNA. Using suitable mixtures of dideoxy A (or C, G or T), and a deoxynucleotide mix, a range of sequence-specific size fragments is generated which can be resolved by electrophoresis to determine the sequence of the synthesized strand. Chain terminations for a given size fragment should only occur in one gel track (A, C, G, or T) in accord and with the specific base pairing of the dideoxynucleotides with the template single-strand. The major drawback is the need to use radioisotopes in a laborious procedure. Automated sequencing instruments will overcome the disadvantage now.

Protein Truncation Test (PTT)

PTT is a rapid, simple and sensitive method for detection of APC gene mutations (Powell et al., 1993; Van et al., 1994). It can be used to scan large regions of genetic materials by detecting translation termination mutations at the protein level. PTT can be used to screen a number of exons simultaneously, allowing screening of exons 1-14 of the APC gene in just one or two tests. RNA is first isolated from peripheral blood mononuclear cells, APC transcripts are converted to

complementary DNA and amplified by reverse transcriptase PCR. The cDNA is amplified with PCR using a special forward primer (that includes a T7 promoter, a eukaryotic translation initiator with an ATG start codon, and a gene-specific 3' sequence designed so that the sequence amplified reads in-frame from the ATG). A coupled transcription-translation system is used to produce polypeptide from the amplified coding sequence, which is then checked for size by gel electrophoresis. A truncated polypeptide gives rise to smaller-sized products, the precise sizes of which can be used to give an estimate of the site of the mutation. There are two features of FAP and the APC gene that make PTT a particularly attractive method. First, most FAP mutations (>95%) result in stop codons, which lead to premature termination of the APC protein. Second, the large size of exon 15, where a high proportion of mutations occurs, means that gDNA can be used as the PCR template, rather than cDNA. The disadvantages of PTT are that it just can not be used to detect nonsense mutations, and it is an experimentally difficult method.

Linkage analysis of the APC gene

The discovery of (CA)n dinucleotide repeats or microsatellites (Litt et al., 1989; Tautz et al., 1989 and Weber et al., 1989) has provided over a thousand new and highly informative genetic markers for human genetic linkage studies. The CA repeat sequences, a kind of variable number of tandem repeat marker (Nakamura Y et al., 1987), are dispersed on the human genome with 50,000 to 100,000 copies. In the flanking region of the APC gene (30-70 kb downstream), there is a tandem repeat CA sequence which shows a length polymorphism with high frequency (Spirio L et al., 1991). In each case the method can be typed conveniently by PCR. Primers are designed from sequences known to flank the repeat regions, permitting PCR amplification of alleles whose sizes differ by integral repeat units. The PCR products can then be size-

fractionated by polyacrylamide gel electrophoresis. A disadvantage of the method is that each allele consists of not just one fragment, but a series of fragments. These "shadow bands" sometimes obscure the position of allelic fragments, which makes genotyping difficult or impossible. This is especially vexing when typing individuals heterozygous for alleles differing in length by only 2 nucleotides.

Table 1-1. CHRPE in FAP

5 types of CHRPE	*3 grades of CHRPE in size*	*Location of CHRPE*
A. oval pigement lesion with pale halo	Grade 1: ≤ 0.5 disk diameter (DD)	Posterior pole
B. small round pigmented dot	Grade 2: 0.5-1.0 disk diameter (DD)	Equatorial
C. large pigmented spot	Grade 3: >1.0 disk diameter (DD)	Peripheral
D. large atropic lesion with or without a pigmented halo		
E. small white dot		

*Baker et al. 1988

Table 1-2. Differences of CHRPE in FAP and unassociated with FAP

	CHRPE in FAP	CHRPE in unassociated with FAP
Features	bilateral, multiple, oval pigmented lesion with a pale halo, small pigmented black dots, depigmented halo containing depigmened lacunae or small satellite lesions	unilateral, solitary, round, flat, circumscribed, hyperpigmented, "bear tracks" or punched-out hyperpigmented or depigmented lacunae lesion
Size	from 50 to 100 μm	from 100 μm to several disk diameters

Table 1-3. Common mutations of the APC gene

Exon	Codon	Nucleotide change	Amino acid change	References
2	51	Deletion of C	Create stop codon	Dobbie et al. 1996
3	78	Deletion of GATA	Create stop codon	Spirio et al. 1993
3	99	CGG-TGG	Arg-Trp	Dobbie et al. 1994
3	121	AGA-TGA	Arg-stop codon	Armstrong et al. 1997
3	139	Deletion of AGAG	Create stop codon	Gismondi et al. 1997
3	141	Deletion of CAT	Create stop codon	Spirio et al. 1993
4	149	Deletion of AA	Create stop codon	Van et al. 1997
4	157	TGG-TAG	Trp-stop codon	Olschwang et al. 1993
4	157	Insertion of T	Create stop codon	Walon et al. 1997
4	159	TAC-TAG	Tyr-stop codon	Marshall et al. 1996
4	168	AGA-TGA	Arg-stop codon	Olschwang et al. 1993
4	169	Deletion of ATAG	Create stop codon	Fodde et al. 1992
4	171	AGT-ATT	Ser-Ile	Van et al. 1997
4	172	Deletion of C	Create stop codon	Dobbie et al. 1994
4	172	Insertion of T	Create stop codon	Armstrong et al. 1997
5	179	Deletion of ACAA	Create stop codon	Nagase et al. 1992
5	185	Deletion of A	Create stop codon	Armstrong et al. 1997
5	196	Deletion of AG	Create stop codon	Paffenholz et al. 1994
5	199	Insertion of G	Create stop codon	Scarano et al. 1997
5	213	CGA-TGA	Arg-stop codon	Miyoshi et al. 1992
5	213	Deletion of A	Create stop codon	Delatycki et al. 1997
5	215	CAG-TAG	Gln-stop codon	Miyoshi et al. 1992
6	232	CGA-TGA	Arg-stop codon	Miyoshi et al. 1992
6	233	CAG-TAG	Gln-stop codon	Smith et al. 1994
6	243	Insertion of AG	Create stop codon	Armstrong et al. 1997
7	266	Insertion of T	Create stop codon	Olschwang et al. 1993
7	267	GGA-TGA	Gly-stop codon	Paffenholz et al. 1994
7	278	CAG-TAG	Gln-stop codon	Bunyan et al. 1995
8	280	TCA-TAA	Ser-stop codon	Koorey et al. 1994
8	280	TCA-TGA	Ser-stop codon	Nishisho et al. 1991
8	283	CGA-TGA	Arg-stop codon	Nagase et al. 1992
8	298	Deletion of TC	Create stop codon	Koorey et al. 1994

Table 1-3. (continued)

Exon	Codon	Nucleotide change	Amino acid change	References
8	302	CGA-TGA	Arg-stop codon	Nishisho et al. 1991
9	357	Insertion of A	Create stop codon	Fodde et al. 1992
9	365	Deletion of CT	Create stop codon	Bunyan et al. 1995
9	405	CGA-TGA	Arg-stop codon	Nagase et al. 1992
9	413	Insertion of T	Create stop codon	Olschwang et al. 1993
9	414	CGC-TGC	Ary-Cys	Nishisho et al. 1991
9	416	Insertion of A	Create stop codon	Olschwang et al. 1993
9	422	GAG-TAG	Glu-stop codon	Olshwang et al. 1993
9	424	CAG-TAG	Gln-stop codon	Marshall et al. 1996
9	435	Deletion of AAG	Create stop codon	Cama et al. 1994
10	440	Deletion of T	Create stop codon	Grobbelaar et al. 1996
10	443	GAA-TAA	Glu-stop codon	Paul et al. 1993
10	455	Deletion of T	Create stop codon	Groden et al. 1991
10	457	Insertion of G	Create stop codon	Nagase et al. 1993
11	742	Deletion of C	Create stop codon	Mandl et al. 1994
11	473	CAG-TAF	Gln-stop codon	Walon et al. 1997
11	494	Deletion of T	Create stop codon	Gismondi et al. 1997
11	495	Deletion of AC	Create stop codon	Van et al. 1997
11	497	Deletion of A	Create stop codon	Dobbie et al. 1994
11	499	CGA-TGA	Arg-stop codon	Olschwang et al. 1993
11	500	TAT-TAG	Tyr-stop codon	Groden et al. 1991
11	503	Insertion of ATTC	Create stop codon	Olschwang et al. 1993
12	520	Deletion of TA	Create stop codon	Armstrong et al. 1997
12	526	Deletion of AGCACTTGTG	Create stop codon	Mandl et al. 1994
12	528	Insertion of A	Create stop codon	Mandl et al. 1994
12	541	CAG-TAG	Gln-stop codon	Miyoshi et al.1992
13	553	TGG-TAG	Trp-stop codon	Nagase et al. 1992
13	554	CGA-TGA	Arg-stop codon	Fodde et al. 1992
13	564	CGA-TGA	Arg-stop codon	Fodde et al. 1992
13	570	Deletion of A	Create stop codon	Van et al. 1997
13	577	Deletion of AAGT	Create stop codon	Armstrong et al. 1997

Table 1-3. (continued)

Exon	Codon	Nucleotide change	Amino acid change	References
13	577	TTA-TAA	Leu-stop codon	Miyoshi et al. 1992
14	584	Deletion of C	Create stop codon	Nagase et al. 1992
14	586	AAA-TAA	Lys-stop codon	Nagase et al. 1992
14	591	Deletion of T	Create stop codon	Marshall et al. 1996
14	592	TTA-TGA	Leu-stop codon	Olschwang et al. 1993
14	593	TGG-TAG	Trp-stop codon	Van et al. 1997
14	593	TGG-TGA	Trp-stop codon	Nagase et al. 1992
14	621	Insertion of A	Create stop codon	Mandl et al. 1994
14	622	TAC-TAA	Tyr-stop codon	Miyoshi et al. 1992
14	624	Deletion of AGAC	Create stop codon	Nagase et al. 1993
14	624	Deletion of CA	Create stop codon	Olschwang et al. 1993
14	625	CAG-TAG	Gln-stop codon	Miyoshi et al. 1992
14	626	Deletion of AACA	Create stop codon	Varesco et al. 1993
14	629	TTA-TAA	Leu-stop codon	Gismondi et al. 1997
14	629	Insertion of A	Create stop codon	Fodde et al. 1992
14	635	Deletion of G	Create stop codon	Armstrong et al. 1997
14	636	Insertion of GT	Create stop codon	Wells et al. 1996
14	638	Deletion of T	Create stop codon	Mandl et al. 1994
14	639	Insertion of A	Create stop codon	Gismondi et al. 1997
14	650	GAG-TAG	Glu-stop codon	Olschwang et al. 1993
14	653	Deletion of AAATCCTAAGAGAC	Create stop codon	Varesco et al. 1993
15	660	Deletion of GT	Create stop codon	Bunyan et al. 1995
15	665	Deletion of TT	Create stop codon	Nagase et al. 1992
15	699	TGG-TGA	Trp-stop codon	Bunyan et al. 1995
15	704	Insertion o T	Create stop codon	Gismondi et al. 1997
15	713	TCA-TGA	Ser-stop codon	Nishisho et al. 1991
15	722	AGT-GGT	Ser-Gly	Stella et al. 1994
15	764	Insertion of T	Create stop codon	Nagase et al. 1992
15	768	Deletion of A	Create stop codon	Bunyan et al. 1995
15	769	Deletion of C	Create stop codon	Paffenholz et al. 1994

Table 1-3. (continued)

Exon	Codon	Nucleotide change	Amino acid change	References
15	779	Insertion of TT	Create stop codon	mandl et al. 1994
15	784	TCT-ACT	Ser-Thr	Miyoshi et al. 1992
15	792	Deletion of A	Create stop codon	Dobbie et al. 1996
15	793	Deletion of G	Create stop codon	Miyashi et al. 1992
15	805	Deletion of AT	Create stop codon	Miyashi et al. 1992
15	805	CGA-TGA	Arg-stop codon	Doobie et al. 1996
15	811	Deletion of ACAA	Create stop codon	Van et al. 1997
15	827	Insertion of AT	Create stop codon	Miyoshi et al. 1992
15	830	Deletion of ACCCAGCTCCTCT	Create stop codon	Stella et al. 1994
15	848	Deletion of TA	Create stop codon	Paffenholz et al.1994
15	855	Deletion of G	Create stop codon	Van et al. 1997
15	856	Deletion of GAATT	Create stop codon	Miyashi et al. 1992
15	902	Deletion of CAGA	Create stop codon	Paffenholz et al. 1994
15	929	Deletion of ACAT	Create stop codon	Armstrong et al. 1997
15	931	Deletion of C	Create stop codon	Nagase et al. 1992
15	932	TCA-TAA	Ser-stop codon	Miyoshi et al. 1992
15	933	Deletion of ACTT	Create stop codon	Armstrong et al. 1997
15	935	TAC-TAG	Tyr-stop codon	Van et al. 1997
15	938	Deletion of TCGG	Create stop codon	Hamzehloei et al.1994
15	954	Deletion of AA	Create stop codon	Van et al. 1997
15	963	Deletion of A	Create stop codon	Gismondi et al. 1997
15	964	Deletion of AATA	Create stop codon	Paffenholz et et al. 1994
15	999	Deletion of TACCCAG	Create stop codon	Nagase et al. 1992
15	1010	Insertion of TA	Create stop codon	Pfaffenhol et al. 1994
15	1032	Deletion of AYGAG	Create stop codon	Nagase et al. 1993
15	1033	Deletion of A	Create stop codon	Van et al. 1997
15	1041	CAA-TAA	Gln-stop codon	Nagase et al. 1992
15	1045	CAG-TAG	Gln-stop codon	Gebert et al. 1994
15	1054	Deletion of TAATA	Create stop codon	Miyashi et al. 1992
15	1055	Deletion of TAGAA	Create stop codon	Armstrong et al. 1997

Table 1-3. (continued)

Exon	Codon	Nucleotide change	Amino acid change	References
15	1060	Deletion of ACAAA	Create stop codon	Miyashi et al. 1992
15	1061	Deletion of CAAA	Create stop codon	Dobbie et al. 1996
15	1061	Deletion of AA	Create stop codon	Gismondi et al. 1997
15	1062	Deletion of GTGA	Create stop codon	Ichii et al. 1993
15	1065	Deletion of AC	Create stop codon	Bapat et al. 1994
15	1079	Deletion of AG	Create stop codon	Armstrong et al. 1997
15	1084	Deletion of AAAC	Create stop codon	Wells et al. 1996
15	1085	Insertion of C	Create stop codon	Van et al. 1997
15	1095	Insertion of A	Create stop codon	Kotze et al. 1996
15	1110	TCA-TGA	Ser-stop codon	Nagase et al. 1992
15	1120	Insertion of G	Create stop codon	Van et al. 1997
15	1134	Deletion o f T	Create stop codon	Dobbie et al. 1996
15	1146	Deletion of C	Create stop codon	Walon et al. 1997
15	1152	CAG-TAG	Gln-stop codon	Van et al. 1997
15	1155	Deletion of AAGA	Create stop codon	Miyashi et al. 1992
15	1179	Insertion of T	Create stop codon	Gismondi et al. 1997
15	1185	Deletion of GA	Create stop codon	Paffenholz et al. 1994
15	1194	TCA-TGA	Ser-stop codon	Gismondi et al. 1997
15	1197	Deletion of AA	Create stop codon	Hamzehloei et al. 1994
15	1207	Deletion of C	Create stop codon	Hamzehloei et al. 1994
15	1211	Insertion of T	Create stop codon	Miyoshi et al. 1992
15	1228	CAG-TAG	Gln-stop codon	Gismondi et al. 1997
15	1233	Deletion of T	Create stop codon	Van et al. 1997
15	1249	TGC-TGA	Cys-stop codon	Miyoshi et al. 1992
15	1259	Deletion of CA	Create stop codon	Bapat et al. 1994
15	1265	Insertion of T	Create stop codon	Gismondi et al. 1997
15	1286	GAA-TAA	Glu-stop codon	v.d.luijt et al. 1994
15	1294	Deletion of A	Create stop codon	Van et al. 1997
15	1301	Deletion of T	Create stop codon	Gismondi et al. 1997
15	1301	Insertion o fC	Create stop codon	Gismondi et al. 1997
15	1307	Deletion of A	Create stop codon	Walon et al. 1997

Table 1-3. (continued)

Exon	Codon	Nucleotide change	Amino acid change	References
15	1309	GAA-TAA	Glu-stop codon	Miyoshi et al. 1992
15	1323	Deletion of A	Create stop codon	Dobbie et al. 1996
15	1323	Insertion of A	Create stop codon	Nagase et al. 1993
15	1328	CAG-TAG	Gln-stop codon	Paul et aj. 1993
15	1336	Deletion of TG	Create stop codon	Van et al. 1997
15	1338	CAG-TAG	Gln-stop codon	Groden et al. 1993
15	1342	TTA-TAA	Leu-stop codon	Van et al. 1997
15	1352	Deletion of G	Create stop codon	Van et al. 1997
15	1357	GGA-TGA	Gly-stop codon	Van et al. 1997
15	1361	Deletion of C	Create stop codon	Marshall et al. 1996
15	1367	CAG-TAG	Gln-stop codon	Hamzehloei et al. 1994
15	1372	Deletion of AC	Create stop codon	Dobbie et al. 1996
15	1382	Insertion of T	Create stop codon	Armstrong et al. 1997
15	1392	TCA-TGA	Ser-stop codon	Doobie et al. 1996
15	1402	Deletion of C	Create stop codon	Dobbie et al. 1996
15	1426	Deletion of C	Create stop codon	Miyashi et al. 1992
15	1450	CGA-TGA	Arg-stop codon	Mori et al. 1993
15	1465	Deletion of AG	Create stop codon	Miyashi et al. 1992
15	1503	Deletion of TCCA	Create stop codon	Armstrong et al. 1997
15	1529	CAG-TAG	Gln-stop codon	v.d.luift et al. 1994
15	1546	Deletion of TGAAA	Create stop codon	v.d.luift et al. 1994
15	1554	Insertion of A	Create stop codon	Gismondi et al. 1997
15	1597	Deletion of T	Create stop codon	v.d.luift et al. 1994
15	1859	Deletion o TTCT	Create stop codon	Gayther et al. 1994
15	1981	Deletion of A	Create stop codon	Scott et al. 1995
15	2570	Deletion of GAATT	Create stop codon	Tamura et al. 1993
15	2643	Deletion of TTAT	Create stop codon	Miyashi et al. 1992
15	2839	CTT-TTT	Leu-Phe	Miyoshi et al. 1992

Table 1-4. Summary of polymorphisms in the APC gene

Exon	Codon	Nucleotide change	Coding change	Enzyme site	Frequency	Refrences
3	84	GGA-GTA	*Gly-Val*		0.01	Poweel et al. 1992
5	+11	C-T	Intronic		0.12	Olschwang et al. 1993
6	235	TTG-TTA	Silent		0.01	Olschwang et al. 1993
7	-24	A-T	Intronic		0.01	Nagase et al. 1992
7	-23	T-C	Intronic		0.01	Nagase et al. 1992
8	+31	A-G	Intronic		0.01	Nagase et al. 1992
11	486	TAC-TAT	Silent	*Rsa*I	0.43/0.57	Groden et al. 199
13	545	GCA-GCG	Silent		0.34/0.66	Miyoshi et al. 1992
13	548	TTG-TTA	Silent		0.01	Miyoshi et al. 1992
14	+8	T-C	Intronic		0.97/0.03	Nagase et al. 1992
15	741	AAT-AAC	Silent		0.01	Nagase et al. 1992
15	870	CCA-TCA	*Pro-Ser*	*Bst*NI	0.98/0.02	Powell et al. 1992
15	1055	ATA-ATT	Silent		0.01	Miyoshi et al. 1992
15	1118	AAT-GAT	Silent		0.01	Nagase et al. 1992
15	1129	TTG-TCG	*Leu-Ser*	*Mbo*II	0.01	Gordon et al. 1994
15	1292	ACG-ATG	*Thr-Met*		0.01	Nagase et al. 1992
15	1304	ATA-GTA	*Iso-Val*		0.01	Miyoshi et al. 1992
15	1359	AAA-AAG	Silent		0.01	Nagase et al. 1992
15	1422	GAT-GAC	Silent	*Dpn*I, *Sau3*AI	0.01	Nagase et al. 1992
15	1493	ACA-ACG	Silent	*BsaJ*I, *Dsa*I	0.35/0.65	Powell et al. 1992
15	1678	GGA-GGG	Silent	*Hgi*AI	0.64/0.36	Nagase et al. 1992
15	1882	GAC-GTC	*Asp-Val*		0.90/0.10	Powell et al. 1992
15	1960	CCA-CCG	Silent	*Msp*I	0.63/0.37	Cottrell et al. 1992
15	2401	CTA-TTA	Silent		0.01	Miyoshi et al. 1992
15	2502	GGT-AGT	*Gly-Ser*		0.01	Miyoshi et al. 1992
15	2568	GGA-GGG	Silent	*Bsr*I	0.96/0.04	Powell et al. 1992

Table 1-5. Advantages and disadvantages of methods for screening mutations in the APC gene

Method	Advantages	Disadvantages
Single Strand Conformation Polymorphism (SSCP)	Simple and rapid	Dose not reveal position of change
Protein Truncation Tes (PTT)	Rapid,sensitive and shows position of mutation	Only for chain terminating mutations, experimentally difficult and expensive
Restriction Enzyme Anaysis	Simple, rapid and cheap	Only for known mutations and quality of enzyme is poor
Denaturing Gradient Gel Electrophoresis (DGGE)	High sensitivity and is nonradioactive	Does not reveal position of mutation, choice of primers is critical,
Chemical Cleavage Mismatch (CCM)	High sensitivity, shows position of change	Toxic chemicals
Restriction Fragment Length Polymorphisms (RFLP)	Simple and rapid	Only for known mutations
Heteroduplex Analysis	Simple	Limited sensitivity, does not reveal position of change

Figure 1-1. A large number of polyps present in the colon in Familial Adenomatous polyposis.

Figure 1-2. "Bear tracks" CHRPE are the typical lesions in cases unassociated with FAP. Flat lesions with large hyperpigmentations are seen on clinical examination.

Figure 1-3. The picture shows CHRPE lesions in a FAP patient. An oval pigment lesion with pale halo (upper arrow) and a small round pigmented dot (down arrow) are observed.

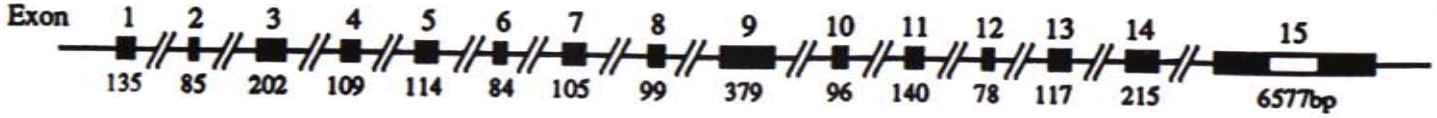

Figure 1-4. The APC gene consists of 15 exons encoding 2843 amino acids. Exon 15 is remarkable for its size, comprising 77% of the protein encoding sequence.

Chapter 2 Study Objectives

1. To identify sequence aberrations in the APC gene in Chinese FAP patients and their family members.
2. To evaluate the presymptomatic diagnostic value of CHRPE in FAP.
3. To investigate the relationship between the APC gene mutations and CHRPE.
4. To study the haplotype associated with FAP and examine its capability for providing accurate diagnostic information and presymptomatic diagnosis of FAP.
5. To formulate the basis for the establishment of an "FAP registry" for Hong Kong.

Chapter 3 Methodology

3.1 Subjects

All study subjects were recruited from patients attending the Eye Clinic, Prince of Wales Hospital, Hong Kong. After the diagnosis of FAP was established, their first-degree and second-degree relatives were at risk of FAP were recruited for study.

3.2 CHRPE analysis

3.2.1 Ophthalmoscopic Examination

All subjects received pupillary dilation and fundal examination by a single observer with indirect ophthalmoscopy and biomicroscopy. The number, size and location of CHRPE were noted by drawing on retinal maps. Clinical data were collected through patient interviews and hospital records.

3.2.2 Diagnostic criteria of CHRPE

CHRPE lesions were classified into 5 types according to the criteria described by Berk et al (1988), and by size into 3 grades. The location of lesions was divided into 3 sites: posterior pole, equatorial and peripheral (Table 1-1). Since small number of CHRPE may occur in normal subjects, the threshold for CHRPE positive is three or more lesions in one or both eyes.

3.3 Materials and Equipments

3.3.1 Enzymes

Taq DNA polymerase was obtained from GIBCO-BRL (Life Technologies, Gaithersburg, MD, USA). The restriction enzyme Hsp92II (10U/ uL) accompanied with 10x buffer and bovine serum albumin (BSA) was purchased from Promega (Promega Corp., Madison, WI, USA).

3.3.2 DNA Markers

HaeIII digested pBR322 DNA marker was obtained from Sigma Chemical Company (St. Louis, MO, USA). 1kb ladder DNA marker was obtained from GIBCO BRL.

3.3.3 Reagent Kits

The QIAamp Blood Kit for DNA extraction was purchased from QIAGEN (Qiagen, USA). Microspin^{Tm}S-300 HR Columns for DNA purification were obtained from Pharmacia Biotech (Det Norske Veitas, Demmark). Thermo Sequenase cycle sequencing kit was obtained from Amersham (Amersham International PLC, Buckinghamshire, UK).

3.3.4 Primers for PCR

Twenty-two primer pairs for PCR were purchased from GIBCO BRL. Table 3-1 summarizes the sequences of these 22 primers.

3.3.5 Chemicals and Reagents

Acrylamide:bis-acrylamide (49:1, 37:1 and 19:1) 40% solution, agarose, ammonium persulfate (AMBS), boric acid, ethidium bromide, ethylenediamine-tetraacetate di-sodium salt (EDTA), formamide, glycerol, sodium dodecyl sulfate (SDS), sucrose, Tris (hydroxymethyl) aminomethane hydrochloride (Tris), Triton X-100, xylene cyanol FF and urea were obtained from Sigma (St. Louis, MO, USA). Ammonium acetate, bromophenol blue, ethanol (absolute), hydrochloric acid, magnesium chloride and mineral oil were from E. Merck (Darmstadt, Germany). The dATP, dCTP, dGTP, dTTP and magnesium chloride for PCR and N,N,N′,N′-tetramethyl ethylenediamine (TEMED) were purchased from GIBCO BRL. Ficoll (type 400) was obtained from Pharmacia (Det Norske Veitas, Demmark). The Kodak XK-1 X-ray film and film developer and fixer were obtained from Eastman Kodak Company (Rochester, NY, USA).

3.3.6 Radioisotopes

α-^{32}P-dCTP (6000 U/mmol, 10 mCi/mL) and γ^{32}P-ATP (>5000 Ci/mmol, 10 mCi/mL) were obtained from Amersham (Amersham International PLC, Buckinghamshire, UK).

3.3.7 Solutions and Buffers

-Agarose gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF and 15% type 400 ficoll)

-Ethanol (70% v/v)

-Ethidium bromide (0.25 ug/mL)

-Nuclei lysis buffer (10 mmol/L Tris pH 8.2, 400 mol/L sodium chloride and 2 mmol/L EDTA)

-Polyacrylamide gel loading buffer (80% formamide, 0.1% bromophenol blue and 0.1% xylene cyanol FF)

-Proteinase K (10 mg/mL, in 1% SDS and 2 mmol/L EDTA)

-Saturated sodium chloride (approx. 6 mol/L)

-Sodium dodecyl sulfate (SDS, 10% w/v)

-10x TAE buffer (10 mmol/L Tris and 1 mmol/L EDTA, pH7.5)

-5x TBE buffer (45 mmol/L Tris-borate, 1 mmol/L EDTA, pH8.3)

3.3.8 Equipment

-Sorvall RC28S centrifuge was obtained from DuPont (Newtown, CT, USA)

-G24 Environmental Incubator Shaker was bought from New Brunswick Scientific Co. (Edison, NJ, USA)

-Response Spectrophotometer was purchased from Gilford, Japan

-Perkin Elmer Cetus Thermal Cycler 480 was purchased from Perkin Elmer Co.(Norwalk, USA)

-Electrophoresis Tank, model Horizon 11.14 was bought from GIBCO BRL (Life Technologies, Gaithersburg, MD, USA)

-Electrophoresis Power Supply EPS 500/400 was obained from Pharmacia (Det Norske Veitas, Demmark)

-UV-Transilluminator at 309 nm was purchased from UVP Inc. (CA, USA)

-Movie Camera HS-CX7 was bought from Mitsubishi Electric Corp (Japan)

-Kodak Wratten 23A gelatin filter was obtained from Sigma (St. Louis, MO, USA)

-Vertical Electrophoresis Tank, model 2 was purchased from GIBCO BRL (Life Technologies, Gaithersburg, MD, USA)

-DC Power supply PS 3000 was bought from Hoefer

-Model 583 Gel Dryer was obtained from BIO-RAD

3.4 Methods

3.4.1 Blood Collection

Blood samples were collected by venipuncture into EDTA tubes from all study subjects. The blood samples were stored at –20 °C for not more than one month prior to DNA extraction.

3.4.2 DNA Extraction

The Salting-out Method

DNA in a blood sample was extracted according to the salting-out method (Miller et al, 1988). A sample of 2.5 mL EDTA blood was transferred to a 15 mL Elkay polypropylene test tube which was then filled with 10mL of freshly prepared sucrose lysis buffer. The sucrose lysis buffer must be kept on ice to lyse the blood cells. The nuclei were sedimented by centrifugation using a Sorvall RC28S centrifuge at 10,000 rpm at 4 °C for 12 minutes. The supernatant was discarded and the pellet washed twice with 10 mL of sucrose lysis buffer followed by centrifugation as above. The pellet was then resuspended in 1.5mL of nuclear lysis buffer followed by addition of 100 µl 10% SDS and 50 µL proteinase K. The nuclear lysate was digested overnight at 37°C in an incubator with constant shaking (G24 Environmental Incubator Shaker). 200 µL of saturated sodium chloride solution was added, and the tube was shaken vigorously for 15 seconds, followed by centrifugation at 10,000 rpm for 12 minutes. The supernatant containing the DNA

was transferred to another 15mL polypropylene tube, and 10 mL of absolute ethanol was added. The DNA was precipitated out by inverting the tube, and then was transferred to a 1.5mL polypropylene tube and washed using 70% ethanol. After drying the DNA under vacuum for 5 minutes, 50 μL of distilled water was added. The DNA was dissolved overnight at 37°C. The DNA sample was then stored at 4 °C.

Method with QIAamp Blood Kit

DNA was extracted from whole blood using the QIAamp Blood Kit (QIAGEN, USA). The procedures were followed according to the protocol in the QIAamp Blood Kit Handbook.

3.4.3 DNA Quantitation

The DNA sample was diluted 20 times (10μL DNA+190μL H_2O), and the absorbance at 260 nm and 280 nm was measured with the Response spectrophotometer. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50ug/ml for double-stranded DNA. The ratio between the readings at 260nm to 280nm provides an estimate of the purity of the nucleic acid. Pure preparations of DNA have OD_{260}/OD_{280} values of 1.8 and 2.0.

3.4.4 Polymerase Chain Reaction (PCR)

Twenty-two different segments (from exon 6 to 15) of the APC gene were amplified by polymerase chain reaction (PCR) using twenty-two primer pairs. Exon 15 was amplified with six PCRs because of its large size. Table 3-1 lists the primers and the sizes of PCR products of the

APC gene used in this study. Each PCR tube contained 3.6 ng genomic DNA, 0.2 mM of each deoxy-nucleotide (dNTP), 0.5 U Taq DNA polymerase, 0.3 μg of each primer, 1x PCR buffer from the manufacturer (GIBCO BRL) and 1.5-2.0 mM magnesium chloride (varies for different primers) in a final volume of 25 μL. The reaction mixture was topped with 50 μL of mineral oil to prevent evaporation during PCR. The PCR was carried out on a thermal cycler. The programme began with a denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C-64°C (depended on different primers) for 1 minute and elongation at 72°C for 7 minutes. If the PCR products were for SSCP analysis, 0.1 μL of α^{32}P-dCTP was added to each reaction mixture prior to the PCR process. PCR optimization of each primer was accomplished by adjusting both the magnesium concentration in the PCR reaction mixture and the annealing temperature in the PCR temperature program.

3.4.5 Agarose Gel Electrophoresis

Horizontal agarose gel electrophoresis was carried out in a gel electrophoresis tank. An agarose gel of 2% was prepared by mixing 1.2g agarose and 60mL 1x TAE buffer, and heating in a microwave oven until the agarose dissolved, then ethidium bromide was added to a final concentration of 0.5μg/mL. Then the solution was poured into an electrophoresis tray for solidification. Comb was placed immediately, the teeth of which would form the sample wells. After the gel was completely set (30-45 minutes at room temperature), the comb was removed carefully. The solidified gel was placed in a tank of 1x TAE buffer. DNA marker and samples mixed with loading buffer were loaded into the wells of the gel. Electrophoresis was then carried out at 80-100 mA until the bromophenol blue migrated the desired distance. The gel was then viewed under an ultra-violet (UV) transilluminator at 309 nm and a photograph taken with a

video camera (Movie Camera HS-CX7) using a Kodak Wratten 23A gelatin filter (Sigma) and video copy processor (p66E Video Copy Processor and K65HM Thermal Paper).

3.4.6 Single Strand Conformation Polymorphism

Polyacrylamide gels (acrylamide:bisacrylamide=49:1) with 5% glycerol and polyacrylamide gels (acrylamide: bisacrylamide=37:1) with 10% glycerol were prepared using a sequencing apparatus (GIBCO BRL). Compositions of the two different polyacrylamide gels are listed in Table 3-2. 25 μL of the ^{32}P-labelled PCR product was mixed with 100 μL of loading dye (95% formamide, 10 mmol/L EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol), denatured at 95°C for 5 minutes and placed immediately on ice. 4 μL of the reaction mixture was loaded on the gel and run in 0.5x TBE buffer in two conditions to increase sensitivity. The single strand conformation polymorphism (SSCP) conditions are listed in Table 3-3. After electrophoresis, the gel was then transferred to a 3MM paper and dried on a gel dryer (Bio-Rad Laboratories). The dried gel was exposed to a Kodak XK-1 X-ray film (Eastman Kodak Co.) at –70°C overnight, and then the film was developed.

3.4.7 Direct DNA Sequencing

Direct DNA sequencing were performed on the study samples showing mobility shift on SSCP using the Thermo Sequenase cycle sequencing kit from Amersham.

Amplification and purification of DNA samples

DNA was amplified using the same primers and same conditions as those for PCR-SSCP analysis. The amplified DNA samples were purified with the use of a Microspin^{Tm}S-300 HR Column from Pharmacia Biotech.

Labeling primers

The primers in the sequencing reactions were labelled at the 3′-end with γ^{32}P-dATP (Amersham). 0.2 μg of the primer was mixed with 1 μL of 10x Kinase reaction buffer, 1 μL of γ^{32}P-dATP, 1 U T4 polynucleotide kinase and 4.8 μL distilled water in a final volume of 10 μL. The mixture was incubated at 37°C for 30 minutes, and then heated at 95°C for 5 minutes to inactivate the kinase.

Cycle sequencing

For the sequencing reaction, 4 tubes labeled A, C, G and T were prepared. The A, C, G and T tubes were added 4 μL of the ddATP, ddCTP, ddGTP and ddTTP termination mix, respectively. A master mix was prepared by mixing 2 μL reaction buffer, 1 μL ^{32}P-labeled primer, 2 μL Thermo sequenase DNA polymerase, 20 ng purified DNA template (3 μL) and 9.5 μL distilled water in a final volume of 17.5 μL, from which 4 μL was added to each of the 4 tubes. The total volume in each reaction was 8 μL. Each sequencing reaction tube was overlaid with one drop of mineral oil and placed in the thermal cycler. The cycling program was carried out using the same temperature program as for PCR. 4 μL of stop solution was added into each sequencing tube.

Denaturing Polyacrylamide Gel Electrophoresis

The polyacrylamide gel solutions were prepared by mixing 25.2g urea, 3 mL 20x Glycerol Tolerant buffer and 9 mL 40% acrylamide solution (acrylamide: bisacrylamide=19:1), adjusting the final volume to 60 mL with deionized water and filtering through Whatman #1 paper. Then 450 μL 10% ammonium persulfate plus (AMPS) and 30 μL tetramethylethylene diamine (TEMED) were added, and the gel was poured and allowed to set. The polyacrylamide gel was prepared in 1x Glycerol Tolerant buffer using a sequencing apparatus (GIBCO BRL). The gel was pre-run at 70 W for half an hour, which should bring the temperature at the surface of the front glass plate to ≥40°C, as measured with a surface thermometer. The sequencing reaction products were denatured at 95°C for 5 minutes, 4 μL of the products was loaded on to the gel, and the gel was run at 60 W for 1.5 to 4.5 hours depending on the fragment size. After electrophoresis, the gels were then transferred to a 3MM paper and dried on a gel dryer (Bio-Rad Laboratories). The dried gel was exposed to a Kodak XK-1 X-ray film (Eastman Kodak Co.) at –70°C overnight, and then the film was developed.

3.4.8 Microsatellite marker

Indirect genotype analysis was performed in 4 of the 6 FAP families by using the microsatellite markers D5S1470, D5S2500, D5S1725, D5S1462 and D5S2501. The remaining 2 families (Family A and Family B) were not analyzed by this method, because Family A had few family members (only 3), and Family B that carried an APC mutation had clear segregation.

Labeling primers

The forward primers were end-labeled with γ^{32}P-dATP. 40 pmol of forward primer was mixed with 2 μL of 10x Kinase reaction buffer, 5 μL of γ^{32}P-dATP, 2 U T4 polynucleotide kinase and 7 μL distilled water in a final volume of 20 μL. The mixture was incubated at 37°C for 30 minutes, and then heated at 95°C for 5 minutes to inactivate the kinase.

Polymerase Chain Reaction (PCR)

A master mix was prepared by mixing 0.3 mM of each deoxy-nucleotide (dNTP), 2.0 U Taq DNA polymerase, 20 pmol labeled forward primer, 40 pmol unlabelled reverse primer, 32 μl of 10 x PCR buffer, 2.0 mM magnesium chloride, 15 μl of 6% dimethylsulfoxide (DMSO), and 172.7 μl deionized water in a final volume of 256 μl. 4 μl of the master mix was added to each PCR tube with 12 ng DNA in a final volume of 5 μl. Each PCR reaction tube was overlaid with one drop of mineral oil. Samples were processed through an initial denaturation step at 94°C for five minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at different temperatures (D5S1470: 58°C, D5S2500: 58°C, D5S1725: 58°C, D5S1462: 60°C and D5S2501: 57°C) for 1 minute and elongation at 72°C for 7 minutes. When the reaction program was finished, 25 μL of loading dye (95% formamide, 10 mmol/L EDTA, 0.1% bromophenol blue and 0.1% xylene cyanol) was added into each reaction tube.

Denaturing Polyacrylamide Gel Electrophoresis

Electrophoresis was performed through a vertical 8% denaturing (8 mol/L urea) polyacrylamide gel (described in Direct DNA Sequencing Section) in 1x Glycerol Tolerant Buffer at 60 W for 3.5 hours. After electrophoresis, the gel was collected, dried, and exposed to a Kodak XK-1 X-ray film at –70°C for 4 hours, and then the film was developed.

Table 3-1. PCR Primers used in this study for amplification of the APC gene

Exon	5'Primer sequence, 5' to 3'	3'Primer sequence, 5' to 3'	PCR fragment size (bp)
Exon 6	GGTAGCCATAGTATGATTATTTCT	CTACCTATTTTTATACCCACAAAC	220
Exon 7	AAGAAAGCCTACACCATTTTTGC	GATCATTCTTAGAACCATCTTGC	250
Exon 8	CATGATGTTATCTGTATTTACC	CTTAGCAAAGTAGTCATGGC	253
Exon 9	GGATATTAAAGTCGTAATTTTGT	CATGCACTACGATGTACACT	458
Exon 10	CATCATTGCTCTTCAAATAACA	CACCAGTAATTGTCTATGTCA	195
Exon 11	TAGATGAGGGTCTTTTTCCTCT	TCATACCTGAGCTATCTTAAG	223
Exon 12	GCTTGGCTTCAAGTTGTCTT	CAGAGTGAGACCCTGCCT	236
Exon 13	TTTCTATTCTTACTGCTAGCATT	ATACACAGGTAAGAAATTAGGA	280
Exon 14	CAACTCTAATTAGATGACCCA	GAGAGTATGAATTCTGTACTT	216
Exon 15A	CAATCATATTATGCCTTTTGTC	GATGGCAAGCTTGAGCCAG	399
Exon 15B	AGTACAAGGATGCCAATATTATG	ACTTCTATCTTTTTCAGAACGAG	346
Exon 15C	AACTACCATCCAGCAACAGA	TCTAGTTCTCCATCATTATCAT	481
Exon 15D	CTGCCCATACACATTCAAACAC	TGTTTGGGTCTTGCCCATCTT	381
Exon 15E	AGTCTTAAATATTCAGATGAGCAG	GTTTCTCTTCATTATATTTTATGCTA	429
Exon 15F	AAGCCTACCAATTATAGTGAACG	AGCTGATGACAAAGATGATAATG	434
Exon 15J	CCCAGACTGCTTCAAAATTACC	GAGCCTCATCTGTACTTCTGC	317
Exon 15K	CCCTCCAAATGAGTTAGCTGC	TTGTGGTATAGGTTTTACTGGTG	351
MCR-A	CAGACTTATTGTGTAGAAGA	CTCCTGAAGAAAATTCAACA	298
MCR-B	AGGGTTCTAGTTTATCTTCA	TCTGCTTGGTGGCATGGTTT	295
MCR-C	GGCATTATAAGCCCCAGTGA	AAATGGCTCATCGAGGCTCA	300
MCR-D	ACTCCAGATGGATTTTCTTG	GGCTGGCTTTTTTGCTTTAC	300

Joanna Groden et al. 1991
Miyoshi et al. 1992a
Kazuo et al. 1994

Table 3-2. Compositions of the two different polyacrylamide gels

	Acrylamide:bisacrylamide=49:1, 5% glycerol	Acrylamide:bisacrylamide= 37: 1, 10% glycerol
Acrylamide:bisacrylamide=49:1	9mL	0
Acrylamide:bisacrylamide=37:1	0	9 mL
5 x TBE	6 mL	6 mL
Glycerol	3 mL	6 mL
Ditiled water	41.52 mL	38.52 mL
10% AMPS	450 uL	450 uL
TEMED	30 uL	30 uL
Final volume	60 mL	60 mL

Table 3-3. Two conditions for running the acrylamide gels in SSCP

Acrylamide gel	Temperature	Power	Time
Acrylamide:bisacrylamide=49:1, 5% glycerol	Room temperature	3 W	16 hours
Acrylamide:bisacrylamide=37:1, 10% glycerol	4°C	6 W	16 hours

Chapter 4 Results

4.1 Study Subjects

4.1.1 FAP index patients

All the FAP patients (mean age 41.2) including 4 males and 6 females were previously diagnosed (mean age 32.9) and had undergone prophylactic colectomy. Extracolonic manifestations (ECM) including desmoids tumours, osteomas, epidermoid cysts and other malignant tumours were not present in all of them. Ophthalmoscopy was performed in 9 of the 10 FAP patients. The remaining one did not have ophthalmoscopic examination because of terminal ill in FAP was at the time of our study.

4.1.2 Family members

Six unrelated independent FAP families were studied, five of them from Guangdong province in southern China and the remaining one from Shanghai in middle China. Family members of 3 generations were available for study in 2 families while only 2 generations were available in 4 families. At least one FAP affected patient was included in each family. Thirty-three family members (mean age 26.4) including 13 males and 20 females from the 6 families were recruited for our study. Sixteen of them consented for sigmoidoscopy and no polyps in the colon were found in all of them. The remaining 17 family members did not consent to endoscopy. Extracolonic manifestations other than CHRPE were not found in any of them. Twenty-nine of

the 33 family members were examined by ophthalmoscopy, but the remaining 4 members did not consent for fundus examination. Genetic study was performed in all these 33 family members.

4.1.3 Control subjects with CHRPE (unassociated with FAP)

CHRPE were observed in 4 study subjects (age range 26-48, 2 male and 2 female) who attended our Eye Clinic for routine ophthalmologic examination. They had no other colonic or extracolonic features of FAP. They also did not have a family history of FAP.

4.1.4 Normal control subjects

Eight spouses (3 males and 5 females) of the affected individuals in the three FAP families were used as normal controls. They were healthy and had no family history of hereditary disease.
All the study subjects are summarized in Table 4-1.

4.2 CHRPE Analysis

4.2.1 CHRPE in FAP index patients

The 9 FAP affected patients had one or more CHRPE lesions (100%). Seven of the 9 had more than three lesions in both eyes (77.8%) and the other 2 patients had only one or two "spot" lesions in the retinae, so the 2 patients were considered negative for CHRPE. Table 4-2 summaries the characteristics of the CHRPE lesions in the 9 FAP patients. Totally 64 CHRPE lesions were observed in the 9 FAP index patients including 19 type A (29.7%), 26 type B (40.6%), 1 type C (1.60%) and 18 type E (28.1%) lesions. Type D lesions were not found. The distribution of these CHRPE lesions was ≤0.5 disk diameter (DD) in 49 of the 64 lesions

(76.56%), 0.5-1.0 DD in 11 lesions (17.19%) and >1.0 DD in 4 lesions (6.25%). Lesions of ≤0.5 DD were the commonest size in FAP affected patients. The locations of the CHRPE lesions were evenly distributed in all three regions of the retina, with 23 (35.94%) in the posterior pole, 22 (34.38%) in the equatorial and 19 (29.68%) in the peripheral.

4.2.2 CHRPE in family members

Out of the 29 relatives, 8 had one or more CHRPE lesions (27.6%) and 2 had more than three (6.9%) (Table 4-3). There were totally 20 CHRPE lesions found in these individuals including 3 type A (15%), 3 type B (15%) and 14 type E (70%). Types C and D lesions were not found. Type E was the most common. Eighteen lesions were ≤ 0.5 DD in size (90%), and 2 lesions were between 0.5-1.0 DD. Of the 19 CHRPE lesions, 7 (35%) were in the posterior pole, 7 (35%) were in the equatorial and 6 (30%) were in the peripheral.

4.2.3 CHRPE in control subjects

CHRPE was observed in the 4 controls with CHRPE during routine ophthalmoscopy. The subjects showed normal vision and visual field. But the CHRPE lesions were different from the CHRPE in FAP; they were solitary (one lesion per subject), big (about 5DD), round and hyperpigmented.

CHRPE was not found in any of the 8 normal control subjects.

4.2.4 Statistical analysis

The ophthalmoscopic data (Table 4-4) were analyzed by the Chi-square test. Confidence limits were calculated to estimate the possible range for sensitivity, specificity and predictive values.

The difference in CHRPE positivity between subjects negative and positive for polyps was significant ($p<0.001$). The cumulative sensitivity of CHRPE was 77.8% (95% confidence limits, 43.88-100%; $p<0.05$) and the specificity was 94.6% (95% confidence limits, 86.95-100%; $p<0.05$). The positive and negative predictive values were 77.8% (95% confidence limits, 43.88-100%; $p<0.05$) and 94.6% (95% confidence limits, 86.95-100%; $p<0.05$) respectively. The distribution of the CHRPE scores in FAP index patients and family members is shown in Figure 4-1.

4.3 PCR optimization

Twenty-two PCR protocols have been established for screening and identification of APC mutations from exon 6 to 15 in all 55 studied subjects (Table 4-5). The PCR protocols were optimized for magnesium concentration in the PCR buffer and annealing temperature in the PCR cycle program. Figure 4-2 shows a sample of the PCR optimization.

4.4 Single strand conformation polymorphism (SSCP)

SSCP protocols were established for screening sequence alterations from wild type in all these PCR products. Eleven of the 22 PCR-SSCP analyses in the APC gene revealed different SSCP patterns; 8 were detected both at room temperature and 4°C, and 3 were observed only at 4°C (Table 4-6). SSCP alternate patterns were not detected in exons 7, 12 and 14 with the conditions so far used.

4.5 Direct DNA sequencing analysis

The SSCP positive conformers were further investigated by direct sequencing of the PCR products. The protocol for direct DNA sequencing analysis of the APC gene was established as described in the methods section. Four nonsense mutations (one of them novel) (Table 4-7), 3 new silent mutations and 4 polymorphisms (Table 4-8) were identified.

4.5.1 Nonsense mutations

3068insA.

The novel mutation is at nucleotide position 3068 in exon 15 of the APC gene. It is an one base pair (A) insertion at codon 1023 (Figure 4-4), designated as 3068insA. The mutation creates a downstream stop codon within 17 nucleotides. This mutation was revealed on SSCP with one extra single strand band with slightly slower mobility from the PCR product of exon 15D (Figure 4-3). This mutation was detected in one FAP index patient (III1) in family A.

Arg216Stop.

The noted mutation is in exon 6 of the APC gene. It is a C→T substitution at nucleotide 646, changing codon 216 from arginine (CGA) to a stop codon (TGA) (Figure 4-6), designated as Arg216Stop or R216X. The mutation was revealed on SSCP with one extra single strand band with slightly slower mobility from the PCR product of exon 6 (Figure 4-5). The mutation was found in one FAP patient (II1) in family D.

Arg283Stop

The noted mutation is in exon 8. It is a C→T substitution at nucleotide 847 changing codon 283 from arginine (CGA) to a stop codon (TGA) (Figure 4-8), designated as Arg283Stop or R283X. It is expected to lead to a truncated and inactive protein. The mutation was revealed on SSCP with one extra single strand band with slightly faster mobility from the PCR product of exon 8 (Figure 4-7). The mutation was detected in 5 subjects in family B, including 4 FAP patients (II1, II5, II7 and III1) and 1 family member (III2).

3183del5

The noted mutation is in exon 15. This is a 5 base pair deletion of ACAAA at nucleotide 3183 in codon 1061 (Figure 4-10), designated as 3183del5. This mutation produced a frameshift and created a stop codon (AAG) within 2 nucleotides. From the PCR product of exon 15E, the 5 base pair deletion was revealed on SSCP with one extra single strand band with faster mobility and one single strand band migrated faster, and also a heteroduplex double strand band (Figure 4-9). The mutation was detected in one FAP affected patient (II8) in family C.

Figure 4-11 is a summary of these 4 nonsense mutations of the APC gene detected.

4.5.2 Novel silent mutations

Ser1050Ser in exon 9

The novel silent mutation was found in exon 9 of the APC gene. It is a T→G change at nucleotide position 1050, the third base of codon 350 (Figure 4-13). The amino acid is unaltered, since both TCT and TCG encode serine. This silent mutation was revealed by SSCP with one

extra single strand band with slower mobility from the PCR product of exon 9 (Figure 4-12). The silent mutation was detected in 2 subjects in family E, one family member (III5) and one normal control (II8).

G→A Transition in exon 10 and intron 10 boundary

This novel silent mutation was found in intron 10. It is a G→A intronic change 5 bp downstream of the splice donor site for exon 10 (Figure 4-15). This silent mutation was revealed by SSCP with one extra single strand band of faster mobility from the PCR product of exon 10 (Figure 4-14). The silent mutation was detected in 6 subjects including one FAP patient and 5 family members in family C.

Thr 4965 Thr in exon 15

This novel silent mutation was found in exon 15J in a normal control (II3) in family A. It is an A→G change at nucleotide position 4965, which is the third base of codon 1655 (Figure 4-17). The amino acid is unaltered, since both ACA and ACG encode threonine. This silent mutation was revealed on SSCP with one extra single strand band with faster mobility from the PCR product of exon 15J (Figure 4-16).

4.5.3 polymorphisms

Exon 11 Polymorphism

A polymorphism was detected in exon 11 of the APC gene. It is a change from C to T at nucleotide position 1458 (Figure 4-19), which does not alter the amino acid (tyrosine). The

polymorphism was revealed by SSCP showing 3 different patterns (Figur 4-18), which signified homozygosity and heterozygosity for the polymorphism. Thirty-one of the 55 study subjects were observed to carry this polymorphism: 22 as heterozygotes, 9 as homozygotes. Frequencies of the rare allele (T) were: 0.35 in FAP patients, 0.29 in family members and 0.75 in normal controls.

Exon 13 Polymorphism

A polymorphism was found in exon 13. It is a change from A to G at nucleotide position 1635 (Figure 4-21) that does not alter the amino acid threonine. Three different SSCP patterns were noted (Figure 4-20), which signified homozygosity and heterozygosity for the polymorphism. The polymorphism occurred in 8 study subjects including 1 FAP patients, 3 family members in 3 different families and 4 normal controls. Frequences of the rare allele were 0.05 in FAP patients, 0.045 in family members and 0.31 in normal controls in this study.

Exon 15 Polymorphisms

Two polymorphisms were found in exon 15 of the APC gene. They were changes from G to A at nucleotide position 4479 and from A to G at nucleotide position 5034 (Figure 4-23, Figure 4-25). Amino acids (serine and threonine) were not altered by these 2 polymorphisms. The polymorphisms were revealed from the 3 patterns detected by SSCP (Figure 4-22, Figure 4-24), that indicated homozygosity and heterozygosity for the polymorphisms. These 2 polymorphisms occurred together in 8 study subjects including 1 FAP index patient, 3 family members in 3 different families and 4 normal controls. They were the same subjects who possessed the exon 13

polymorphism. Frequences of the rare alleles were the same in the 2 polymorphisms, 0.05 in FAP index patients, 0.045 in family members and 0.31 in normal controls.

4.6 Haplotype Analysis

Haplotype analysis was carried out using 5 microsatellite markers in four FAP families. The order of these markers in the haplotypes is: D5S1470-D5S2500-D5S1725-APC-D5S1462-D5S2501. These markers are tightly linked to the APC gene. Table 4-9 summarises the characteristics of these markers. These 5 microsatellite markers were informative because they revealed a large number of alleles (from 7 to 5) (Figure 4-26-29) and high PIC (polymorphism information content) values (from 0.567 to 0.838) in this study.

4.7 Family studies

4.7.1 Family A (Figure 4-30)

The proband III1 of the family was a 29-years-old woman, she was affected with FAP and had a colectomy performed 8 years ago. She was heterozygous for the 3068insA mutation (novel mutation) (Figure 4-4) that leads to a truncated and inactive protein in the APC gene, and her eyes showed 15 CHRPE lesions in the retinae. Her mother II2 was the only family member examined, she did not carry the APC mutation and presented no CHRPE lesions in the retina.

Because the father of the proband could not join the study and the proband had no descendants, so it was difficut to continue to study FAP in this family.

4.7.2 Family B (Figure 4-31)

It is a typical FAP family in which FAP was observed in three generations. Proband (II1) of the family was a 49-year-old woman who manifested polyposis coli and had a colectomy performed 10 years ago. In the proband, seven small CHRPE lesions were observed in both eyes and a stop mutation (R283X) was detected in exon 8 (Figure 4-18). Her mother died of FAP several years ago. The R283X mutation was also detected in the proband's brother (II5), sister (II7) and 2 children (III1, III2); among them II5, II7 and III1 had been diagnosed clinically and the affected colons had been surgically removed. The CHRPE lesions were found in her 2 sisters (II7, II9) and a child (III1). Since the subject (III2) inherited the APC mutation from her mother (II1), so she is at high risk and has been recommended to have endoscopy. The subject (II5) was a FAP patient and carried the APC mutation, but he only showed 2 CHRPE lesions in the retina. In addition, although subject (II9) had 5 CHRPE lesions in both eyes and didn't carry the mutation in the APC gene, it was recommended her colon be examined annually.

4.7.3 Family C (Figure 4-32)

The proband of the family, II8, was a 36-year-old woman with FAP who had undergone colectomy several years previously. Ophthalmologic examination revealed lesions characteristic of CHRPE. Her eyes showed 16 small CHRPE lesions (≤ 1.0 DD) and 2 large lesions (>1.0 DD) in the retinae. In addition, a 5 base pair deletion was found at codon 1061 (3183del5) in exon 15

(Figure 4-10) in the proband. But no family members had any CHRPE lesions except II1, the proband's brother, who showed 5 small CHRPE lesions in both eyes. However, II1 showed normal endoscopy and didn't carry the APC mutation. APC mutations were also not detected in all the family members. We detected the novel silent mutation (Intron10G/A) in 6 stubjects in this family including one FAP patient and 5 family members. In the haplotype analysis, the offsprings shared the alleles of the parents. We were not able to determine which haplotype in the patient (II8) was associated with disease because II8 was the only FAP patient in this family and her mother (I2) had died of unknown causes, so sporadic case also can not be eliminated.

4.7.4 Family D (Figure 4-33)

The proband of the family was a 64 year old woman (II1), on whom colectomy was performed several years ago. She was heterozygous for the R216X mutation (Figure 4-6) and showed 5 CHRPE lesions in the both eyes. But the APC mutation and CHRPE lesions were not detected in all her 3 children. The haplotype that is associated with disease in this family can not be determined because the family size was small, consisting of only 3 members, and II1 was the only clinically defined patient in the family. The three offsprings inherited the same haplotype (4-2-3-5-2) from their mother, they showed normal endoscopy and didn't carry the APC mutation, so they are apparently at low risk. Because the parents of II1 had already died of unknown causes, the possibility cannot be eliminated that II1 was a sporadic case.

4.7.5 Family E (Figure 4-34)

This family included three generations and 4 FAP patients, 2 of whom died several years ago. The proband II3 was a 55-year-old male FAP patient whose affected colon was removed 9 years ago. His mother and a sister died of FAP in 13 and 9 years ago. His brother II9 was 28 year old and also was FAP affected, but unfortunately, he was terminally ill with FAP at the time of our study. However, both mutations in the APC gene and CHRPE lesions in the retina were not detected in all the FAP patients and family members in this family. In the haplotype analysis, the haplotype (2-5-6-4) associated with disease had been determined in two FAP patients (II3 and II9). The offspring III1 inherited the haplotype associated with disease from her father, and was therefore at high risk. III1 should be recommended for endoscopy.

4.7.6 Family F (Figure 4-35)

The proband II2 of the family was a 43-year-old woman with FAP. The affected colon was removed 10 years ago. Her eyes showed 4 CHRPE lesions, but mutations in the APC gene were not detected in this study. In addition, CHRPE lesions and APC mutations were not found in any of the family members in this family. The haplotype associated with disease can not be determined becaues the family was too small and the proband was the only FAP patient in this family. However, the haplotype with disease maybe was 6-7-4-6, because the another haplotype (3-5-5-3) of the proband was also presented in her unaffected sister (II3). The proband's daughter (III1) inherited the suspicious haplotype with disease. The possibility that the proband was a sporadic case also can not be elimited because there were no informations about FAP from her parents.

Table 4-1. Study subjects

Subject	Total No.	Male	Female	Age (range)	E. No.	O. No.	G. No.
FAP index patients	10	4	6	41.2 (28-64)	10	9	10
Family members	33	13	20	26.4 (2-88)	16	29	33
Controls with CHRPE only	4	2	2	36 (26-48)	4	4	4
Normal controls	8	3	5	42.8 (21-69)	8	8	8

E. No.: the number of subjects examined by endoscopy
O. No.: the number of subjects examined by ophthalmoscopy
G. No.: the number of subjects examined by genetic study

Table 4-2. Summary of FAP, CHRPE and APC mutations in all the 10 FAP patients in this study

Patients	FAP	CHRPE											APC mutations
		Number of lesions	Type					Size			Localization			
			A	B	C	D	E	<0.5 DD	0.5-1.0 DD	>1.0 DD	PP	E	P	
AIII1	+	15	9	5	1	0	0	8	7	0	6	5	4	3068insA*
BII1	+	7	0	1	0	0	6	7	0	0	1	4	2	R283X
BII5	+	2	0	0	0	0	2	2	0	0	1	1	0	R283X
BII7	+	7	0	1	0	0	6	6	1	0	2	3	2	R283X
BIII1	+	5	0	4	0	0	1	5	0	0	2	2	1	R283X
CII8	+	18	7	10	0	0	1	15	1	2	10	6	2	3183del5
DII1	+	5	3	2	0	0	0	1	2	2	0	0	5	R216X
EII3	+	1	0	1	0	0	0	1	0	0	0	1	0	No detected
EII9*	+	-			-				-			-		No detected
FII2	+	4	0	2	0	0	2	4	0	0	1	0	3	No detected
Total	-	64	19	26	1	0	18	49	11	4	23	22	19	-

*Ophthalmoscopy was not performed on EII9 because he was terminally ill with FAP at the time of our study.
*3068insA is a novel mutation of the APC gene.

Table 4-3. Summary of CHRPE and APC sequence alterations in all the 33 family members in this study

Family members	CHRPE												APC sequence alterations
	Number of lesions	Type					Size			Localization			
		A	B	C	D	E	<0.5 DD	0.5-1.0 DD	>1.0 DD	PP	E	P	
AII2	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T, 1635A/G, 4479G/A,
BII3	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
BII9	5	0	0	0	0	5	5	0	0	0	2	3	1458C/T
BII10	2	0	2	0	0	0	2	0	0	1	0	1	1458C/T
BIII2	2	0	0	0	0	2	2	0	0	0	1	1	1458C/T, R283X
BIII3	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
BIII4	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
BIII5*				-				-			-		1458C/T
CI1	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T, Intron10G/A
CII1	5	1	1	0	0	3	3	2	0	4	1	0	Intron10G/A
CII3	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T, Intron10G/A
CII5	0	0	0	0	0	0	0	0	0	0	0	0	Intron10G/A
CII6	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T, Intron10G/A
CIII1	1	0	0	0	0	1	1	0	0	0	1	0	1458C/T
CIII2	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
CIII3	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
CIII4	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
DIII1	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T, 1635A/G, 4479G/A,
DIII2	2	2	0	0	0	0	2	0	0	1	1	0	
DIII3	0	0	0	0	0	0	0	0	0	0	0	0	
EI1	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
EII5	0	0	0	0	0	0	0	0	0	0	0	0	
EII7	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
EIII1	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
EIII2	0	0	0	0	0	0	0	0	0	0	0	0	1635A/G, 4479G/A, 5034A/G
EIII3*				-				-			-		1458C/T
EIII4*				-				-			-		
EIII5*				-				-			-		1458C/T, 1050T/G
FII3	0	0	0	0	0	0	0	0	0	0	0	0	1458C/T
FIII1	1	0	0	0	0	1	1	0	0	1	0	0	
FIII2	0	0	0	0	0	0	0	0	0	0	0	0	
FIII3	2	0	0	0	0	2	2	0	0	0	1	1	
FIII4	0	0	0	0	0	0	0	0	0	0	0	0	

*Ophthalmoscopic examination was not performed on these family members

R283X is a nonsense mutation,: 1050T/G and Intron10G/A are novel silent mutations; 1358C/T, 1635A/G, 4479G/A and 5034A/G are polymorphisms detected in this study

Table 4-4. Summary of CHRPE lesions detected in this study

Subjects	The number of subjects	The number of CHRPE positive	The number of CHRPE negative
FAP patients	9	7	2
Family members	29	2	27
Normal controls	8	0	8

Table 4-5. Optimial conditions for amplifications of different fragments of the APC gene

Primers	Location	$MgCl_2$ concentration	Annealing temperrature	Size of product (bp)
Primer 6	Exon 6	2.0mM	59°C	220
Primer 7	Exon 7	2.0mM	64°C	250
Primer 8	Exon 8	2.0mM	58°C	253
Primer 9	Exon 9	2.0mM	58°C	458
Primer 10	Exon 10	2.0mM	58°C	195
Primer 11	Exon 11	1.5mM	58°C	223
Primer 12	Exon 12	1.0mM	60°C	236
Primer 13	Exon 13	2.0mM	58°C	280
Primer 14	Exon 14	2.0mM	58°C	216
Primer 15A	Exon 15	1.5mM	60°C	399
Primer 15B	Exon 15	2.0mM	62°C	346
Primer 15C	Exon 15	2.0mM	58°C	481
Primer 15D	Exon 15	1.5mM	64°C	381
Primer 15E	Exon 15	2.0mM	60°C	429
Primer 15F	Exon 15	2.0mM	64 °C	434
Primer 15J	Exon 15	1.5mM	64 °C	317
Primer 15K	Exon 15	2.0mM	64 °C	351
MCR-A	Exon 15	1.5mM	57 °C	298
MCR-B	Exon 15	1.5mM	58 °C	295
MCR-C	Exon 15	1.5mM	60 °C	300
MCR-D	Exon 15	1.5mM	57 °C	300

Table 4-6. The 11 SSCP positive conformers in the 2 conditions.

Exon	Codon	5% glycerol, acry: bis=49:1, at RT	10% glycerol, acry: bis=37:1, at 4°C
6	216	Not detectable	Detectable
8	283	Detectable	Detectable
9	350	Detectable	Detectable
Intron 10		Detectable	Detectable
11	486	Detectable	Detectable
13	545	Not detectable	Detectable
15	1023	Not detectable	Detectable
15	1061	Detectable	Detectable
15	1493	Detectable	Detectable
15	1655	Detectable	Detectable
15	1678	Detectable	Detectable

Table 4-7. Mutations in the APC gene identified in this study.

Name	Exon	Codon	Nucleotide change	Amino acid change	References
Arg216Stop	6	216	C→T at 646	Arg→stop codon	Miyaki M, 1994
Arg283Stop	8	283	C→T at 847	Arg→stop codon	Olschwang, 1993
3068insA	15	1023	Insertion of A at 3068	Create stop codon	Novel
3183del5	15	1061	Deletion of ACAAA at 3183	Create stop codon	Miyoshi Y, 1992

Table 4-8. Summary of silent mutations and polymorphisms in the APC gene detected in this study

Mutation	Exon	Codon	Nucleotide change	Rare allele	Coding change	A	B	C	References
1050T/G	9	350	T→G	G	Silent	0	0.015	0.06	Novel
Intron 10 G/A	Intron 10		G→A	A	Intronic	0	0.061	0	Novel
1458C/T	11	486	C→T	T	Silent	0.35	0.29	0.75	Groden, 1991
1635A/G	13	545	A→G	G	Silent	0.05	0.045	0.31	Miyoshi, 1992
4479G/A	15	1493	G→A	A	Silent	0.05	0.045	0.31	Powell, 1992
4965A/G	15	1655	A→G	G	Silent	0	0	0.06	Novel
5034A/G	15	1678	A→G	G	Silent	0.05	0.045	0.31	Nagase, 1992

A: The frequency of the rare allele in the 10 FAP patients
B: The frequency of the rare allele in 33 the family members
C: The frequency of the rare allele in 8 the normal controls

Table 4-9. Microsatellite markers used in this study

Marker	Locus	Size range (bp)	Alleles* (frequencies*)	Heterozygosity	PIC*
D5S1470	GATA7C06	197	8 (0.01, 0.07, 0.07, 0.40, 0.15, 0.1, 0.1, 0.1)	0.838	0.776
D5S2500	GATA67D03	155	7 (0.16, 0.20, 0.16, 0.08, 0.18, 0.14, 0.08)	0.921	0.838
D5S1725	GATA89G08	189	6 (0.01, 0.26, 0.07, 0.10, 0.23, 0.33)	0.714	0.747
D5S1462	GATA3H06	271-241	6 (0.028, 0.07, 0.082, 0.19, 0.15, 0.48)	0.757	0.687
D5S2501	GATA68A03	309	4 (0.23, 0.52, 0.23,0.02)	0.719	0.567

PIC*: polymorphism information content. A marker with a PIC of 0 is never informative, one with a PIC of 1 is always informative. The PIC for an autosomal marker is calculated by the formula: $PIC = 1-\sum_{i=1}^{n} p_i^2-\sum_{i=1}^{n}\sum_{j=i+1}^{n} 2p_i^2p_j^2$, where $p_{Ii}$is the frequency of the allele.

Alleles*: the numbers of different alleles detected using the markers in this study

Frequencies*: the frequences of different alleles

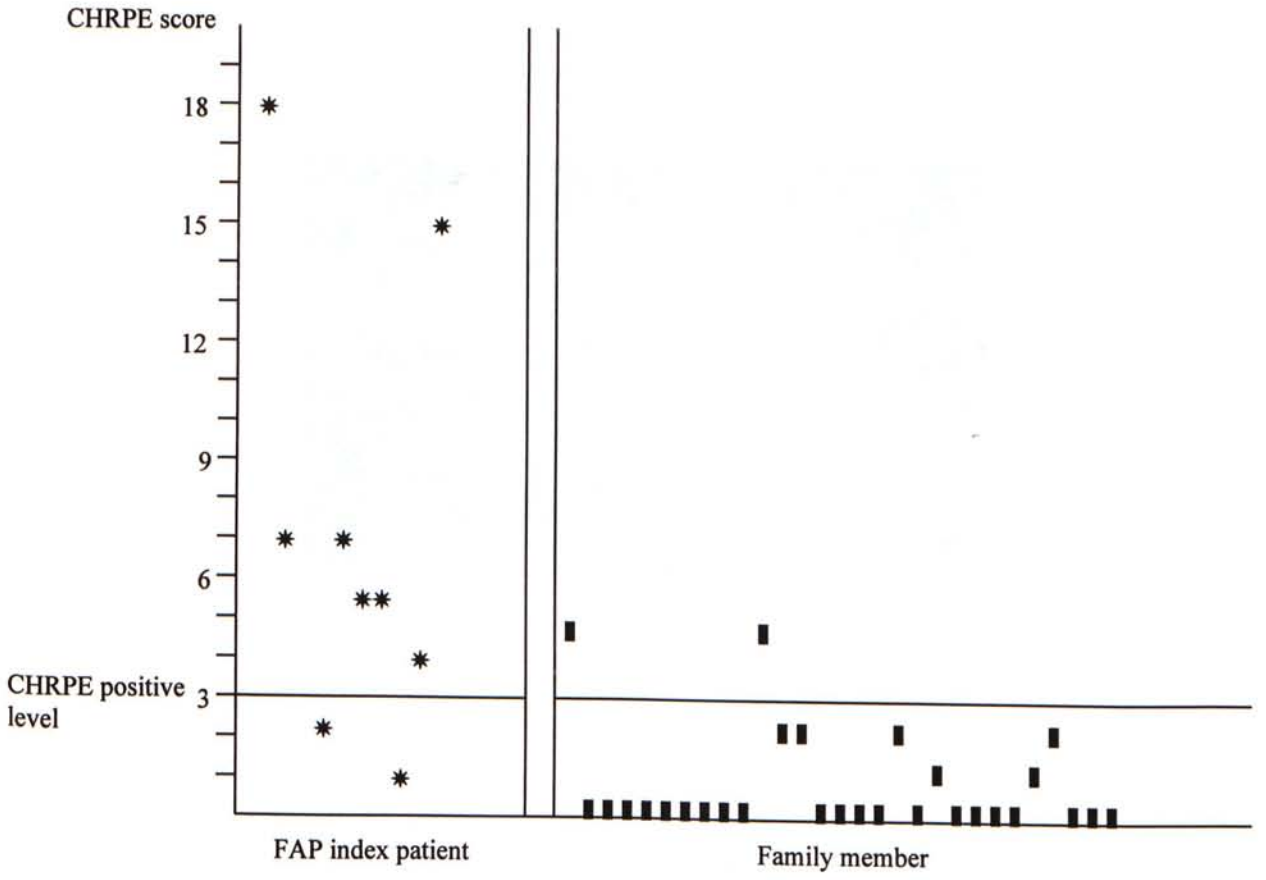

Figure 4-1. Distribution of the CHRPE scores in FAP index patients and family members (✱: FAP index patient, ▮: FAP family member) in this study.

Figure 4-2. An example of amplification of a 481 base pair FAP DNA fragment of the PCR. Lane 1: pBR332 DNA marker; Lane 2: the control without DNA; Lane 3→6: different DNA samples.

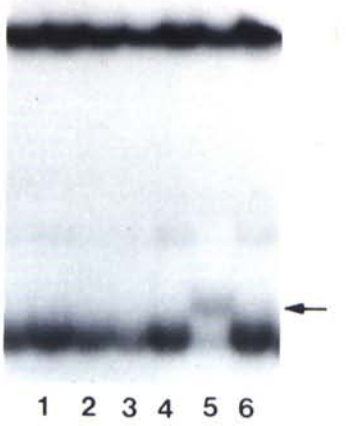

Figure 4-3. SSCP analysis in exon 15D (Table 4-5) of the APC gene. A mutant band (lane 5) presents the FAP patient (III1) in family A. Lane 1→4 and 6 show normal bands. The arrow indicates the position of the abnormal band. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 37:1) containing 10% glycerol at 4°C.

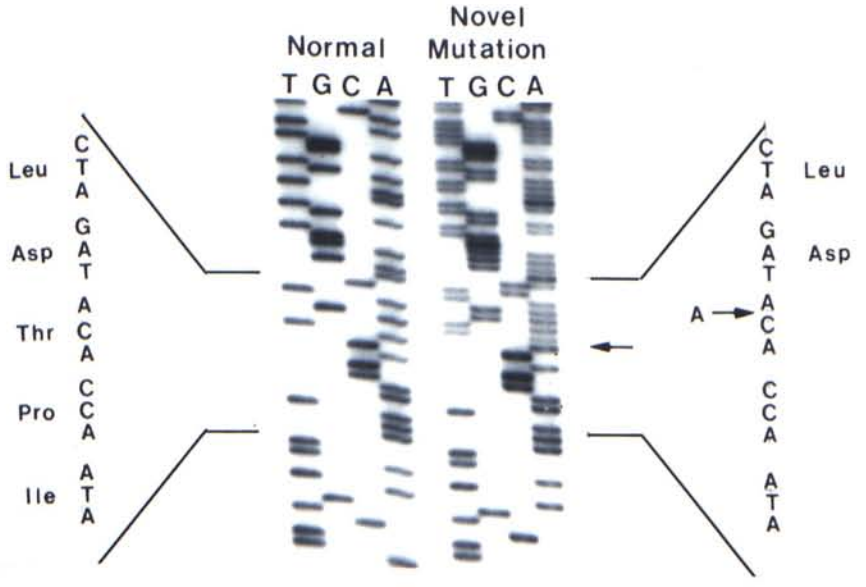

Figure 4-4. Sequencing analysis of mutation in exon 15D of family A. One base pair (A) insertion is detected at nucleotide position 3068 in the FAP patient (AIII1). This is a novel mutation and creates a stop codon 17 nucleotides downstream.

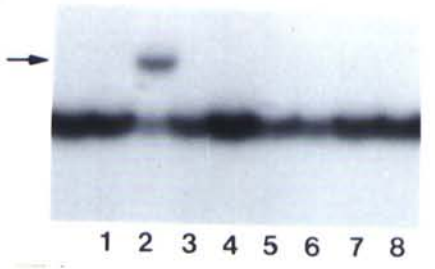

Figure 4-5. SSCP analysis in exon 6 of the APC gene. Mutant band (lane 2) presents the FAP patient (II1) in family D. Lane 1, 3→5 show the normal bands. The arrow indicates the location of the abnormal band. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 37:1) containing 10% glycerol at 4°C

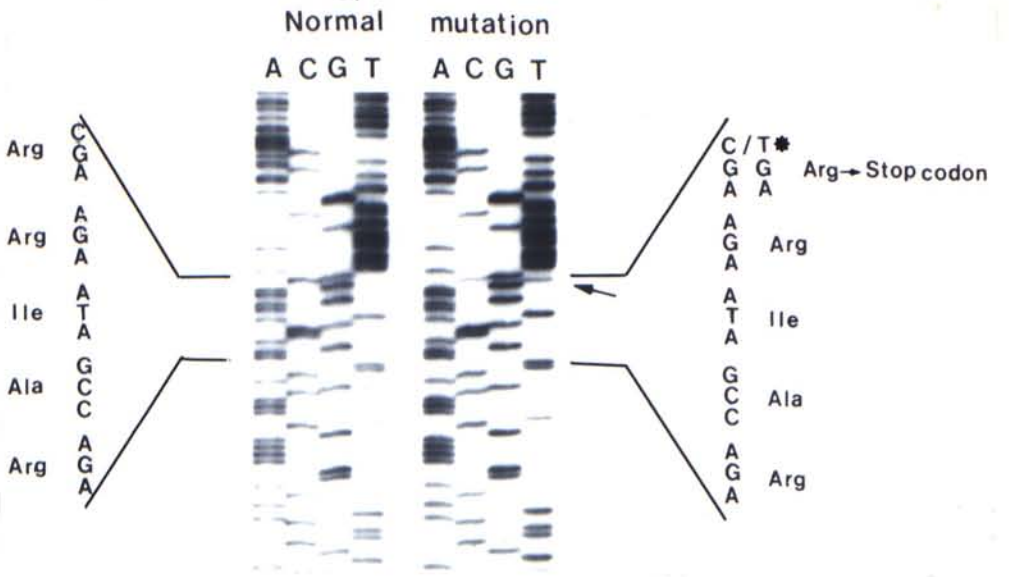

Figure 4-6. Sequencing analysis of the amplified exon 6 in the FAP patient (DII1). Heterozygosity for CGA→TGA is detected at codon 216 of the APC gene. This mutation results in a stop codon at the same position.

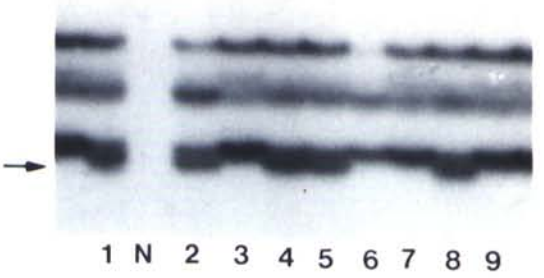

Figure 4-7. SSCP analysis in exon 8 of the APC gene. Mutant bands (lane 1, 2, 4, 5 and 8) present in 4 FAP patients and one family member in family B. Lane 3, 6. 7 and 9 show the normal bands. Lane N is a control without DNA. The arrow indicates location of the abnormal band. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

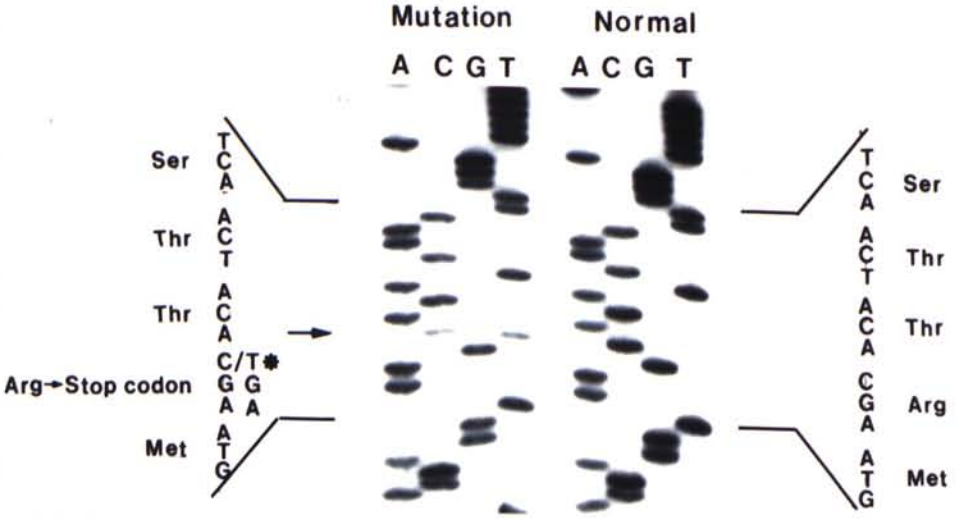

Figure 4-8. Sequencing analysis of exon 8 of Family B. Heterozygosity for CGA→TGA is detected at codon 283 of the APC gene in 4 FAP patients (BII1, BII5, BII7 and BIII1) and one family member (BIII2). This mutation results in a stop codon at the same position.

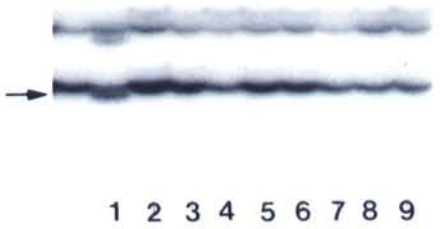

Figure 4-9. SSCP analysis in exon 15E (Table 4-5) of the APC gene. Mutant band (lane 1) presents in one FAP patient (CII8) in family C. Lanes 2→9 are normal samples. The arrow indicates the location of the abnormal band. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

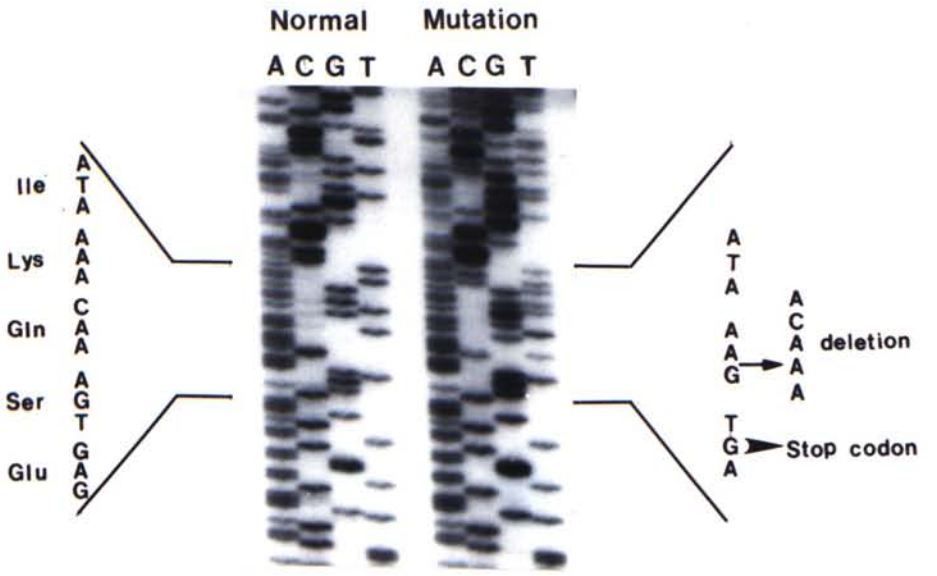

Figure 4-10. Sequencing analysis of exon 15E in the FAP patient (CII8). A deletion of 5 base pairs is detected at codon 1061 of the APC gene. This mutation creates a stop codon in 2 nucleotides downstream.

Figure 4-11. The four nonsense mutations of the APC gene detected in this study are showed above. 3068insA is a novel mutation.

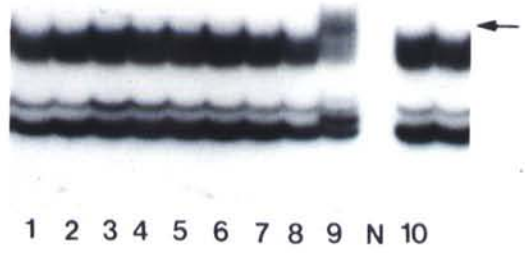

Figure 4-12. SSCP analysis of exon 9 fragment of the APC gene. Mutant band (lane 9) appeared in one family member (EIII5) in family E. Lanes 1→8 and 10 show the normal bands. Lane N is the control without DNA. The arrow indicates the location of the abnormal band. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

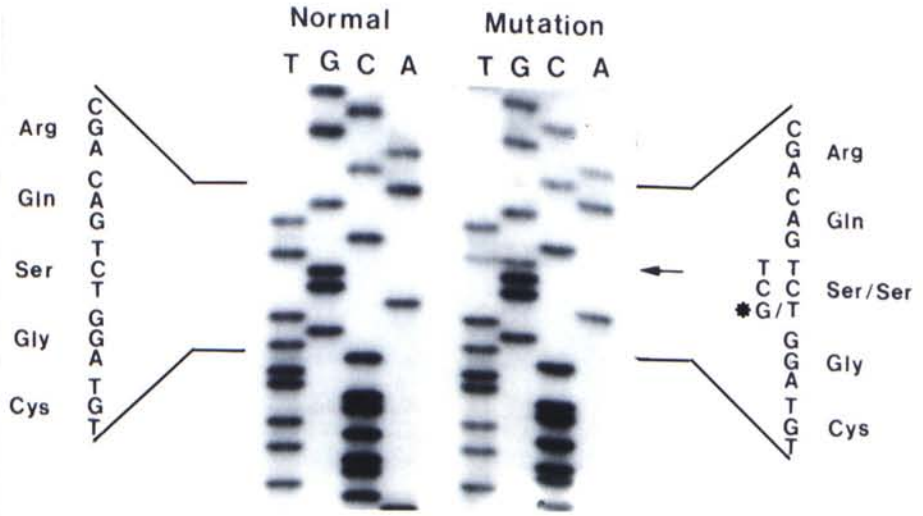

Figure 4-13. Direct sequencing of exon 9 in the family member (EIII5). Heterozygosity for TCT→TCG (samesense mutation) is detected at codon 350 of the APC gene. This is a novel silent mutation.

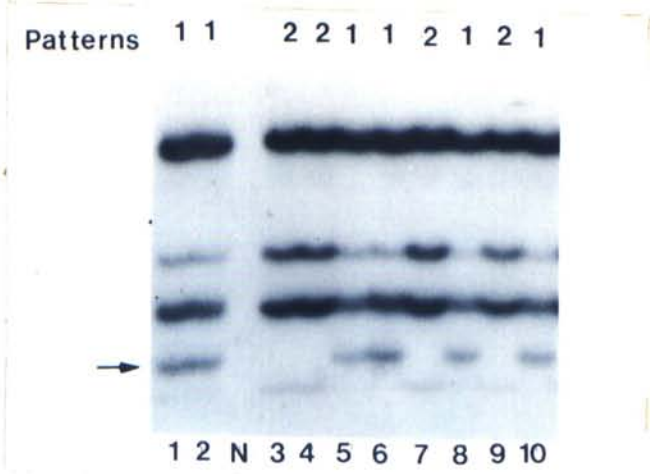

Figure 4-14. SSCP analysis of exon 10 of the APC gene. Two patterns appeared in family C. The arrow indicates the pattern 1. Lane N is control without DNA. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

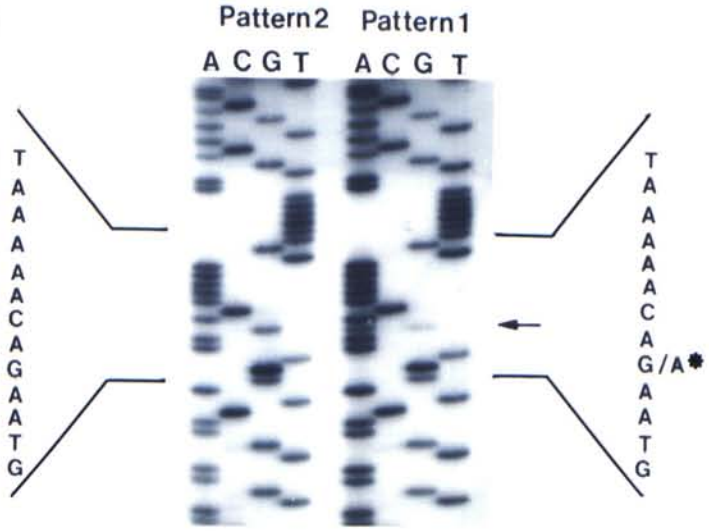

Figure 4-15. Direct sequencing analysis of exon 10 for the 2 patterns. Heterozygosity for G→A is detected in intron 10 of the APC gene in the pattern 1. This is a novel silent mutation.

1 2 3 4 5 6 7 8 9 10 11

Figure 4-16. SSCP analysis of exon 15J (Table 4-5) of the APC gene. Mutant band (lane 8) presents in a normal control (II3) in family A. The arrow indicates the location of the abnormal band. Lane 1→7, 9→11 show the normal bands. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

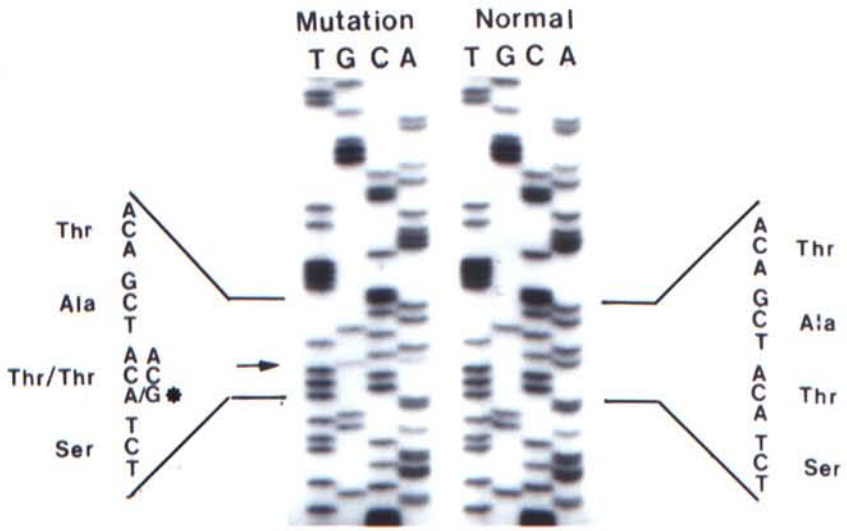

Figure 4-17. Direct sequencing analysis of exon 15J (Table 4-5) for the normal control. Heterozygosity for ACA→ACG (samesense mutation) is detected at codon 1655 of the APC gene. This is a novel silent mutation.

Figure 4-18. SSCP analysis of exon 11 of the APC gene. Three patterns were observed. Pattern 1: lane 1→2, 6→9, and 11→12. Pattern 2: 3→4, 10 and 13→16. Pattern 3: lane 5. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

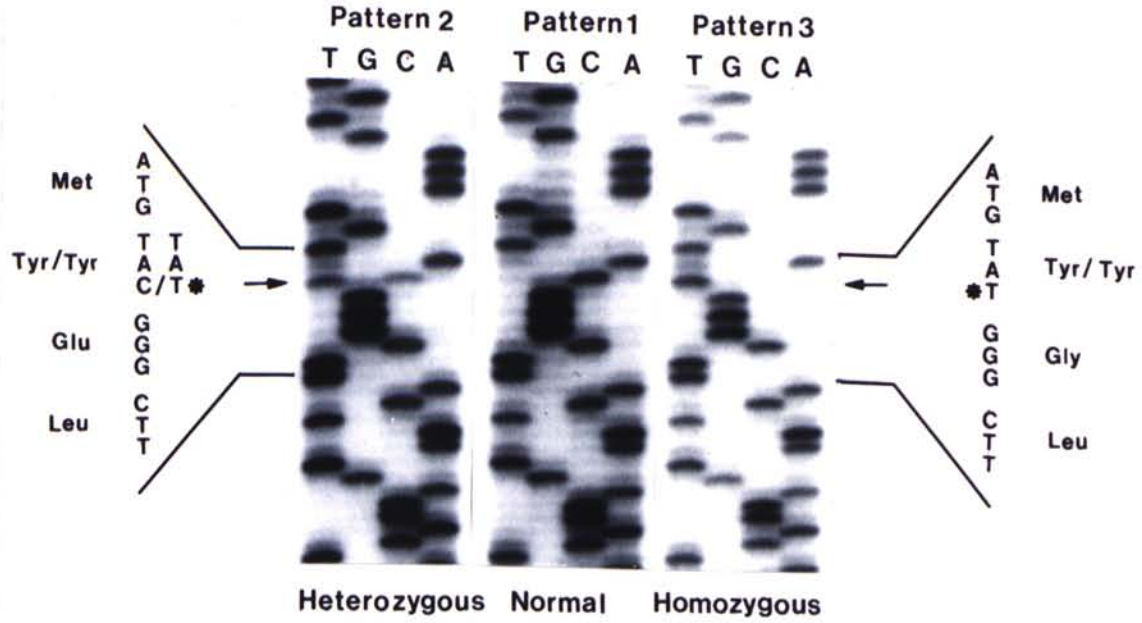

Figure 4-19. Direct sequencing analysis of exon 11 for the 3 SSCP patterns (Figure 4-18). Heterozygosity for TAC→TAT (samesense mutation) is detected in pattern 2 and homozygosity for TAC→TAT in pattern 3. Pattern 1 is the normal pattern.

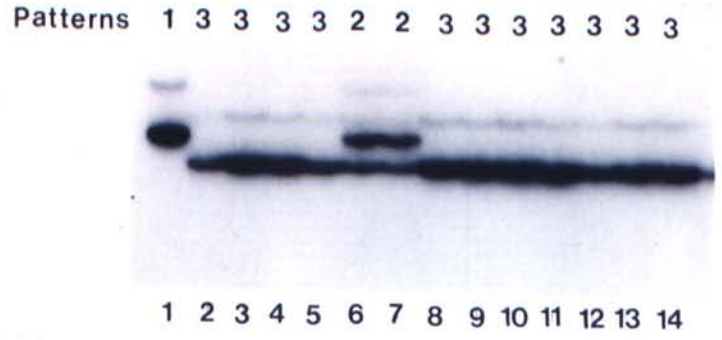

Figure 4-20. SSCP analysis of exon 13 of the APC gene. Three patterns are noted. Pattern 1: lane 1. Pattern 2: lane 6, 7. Pattern 3: lane 2→5 and 8→14. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

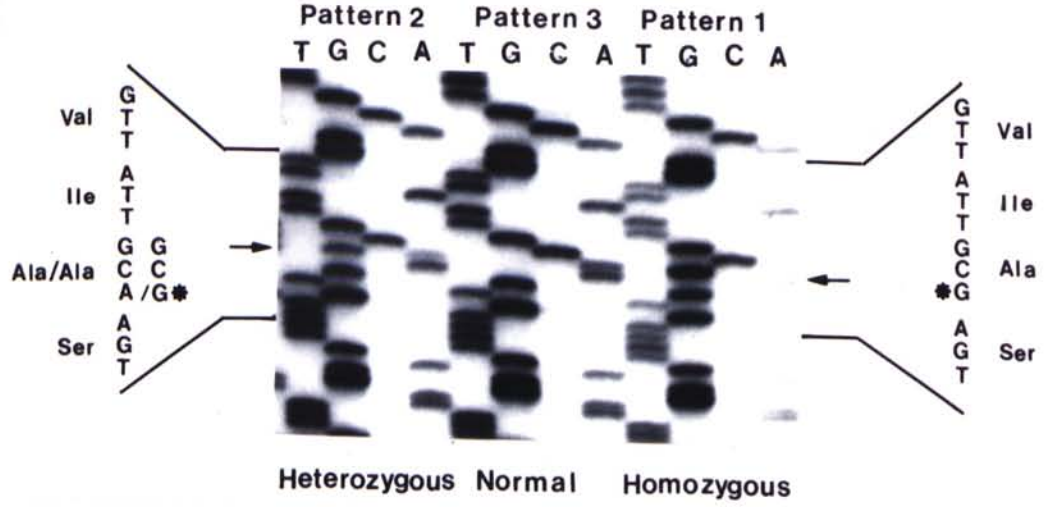

Figure 4-21. Direct sequencing analysis of exon 13 for the 3 patterns. Heterozygosity for GCA→GCG (samesense mutation) is detected in pattern 2 and homozygosity for GCA→GCG in pattern 1. Pattern 3 is the normal pattern.

Figure 4-22. SSCP analysis of exon 15 MCR-C (Table 4-5) of the APC gene. Three patterns are observed. Pattern 1: lane 1→2, 4→6 and 9→10. Pattern 2: lane 3. Pattern 3: lane 7→8. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

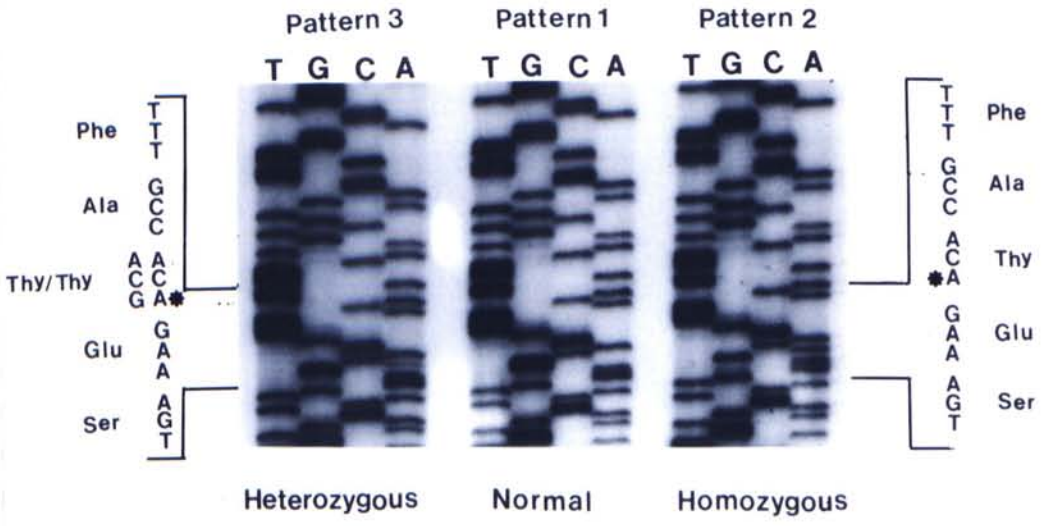

Figure 4-23. Direct sequencing analysis of exon 15 (MCR-C) (Table 4-5) for the 3 patterns. Heterozygosity for ACG→ACA (samesense mutation) is detected in pattern 3 and homozygosity for ACG→ACA in pattern 2. Pattern 1 is the normal pattern.

Figure 4-24. SSCP analysis of exon 15 K (Table 4-5) of the APC gene. Three patterns are noted. Pattern 1: lane 1-2, 4-6 and 9. Pattern 2: lane 3. Pattern 3: lane 7-8. Lane N is the control without DNA. SSCP was performed with 6% polyacrylamide gel (acrylamide: bis-acrylamide = 49:1) containing 5% glycerol at room temperature.

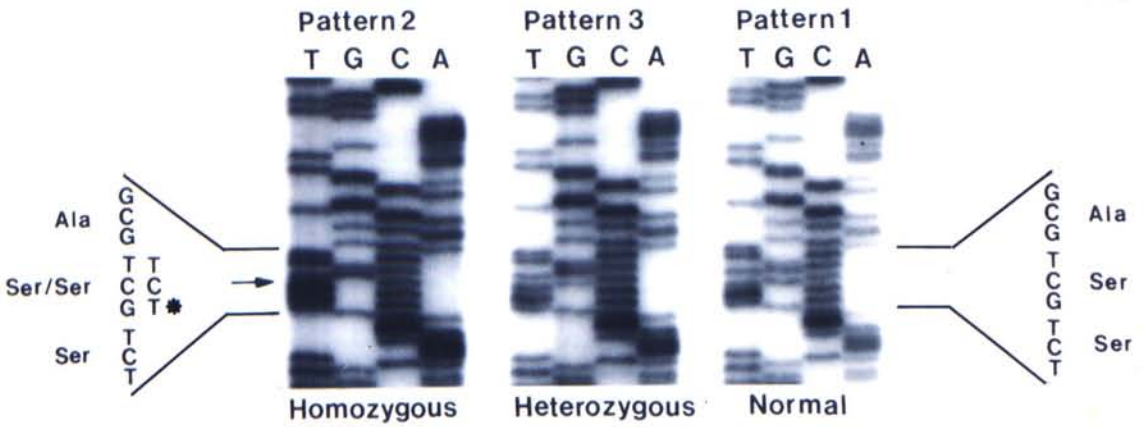

Figure 4-25. Direct sequencing analysis in exon 15 K from the 3 patterns. Heterozygosity for TCG→TCT (samesense mutation) is detected in pattern 3 and homozygosity for TCG→TCT in pattern 2. Pattern 1 is the normal pattern.

Figure 4-26. Haplotype analysis using microsatellite marker (D5S2500) in family C. Types of alleles are showed with the arrows. All subjects are heterozygous in the alleles typed.

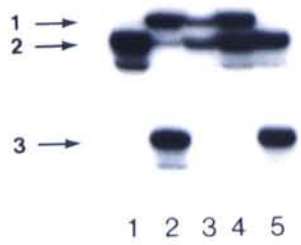

Figure 4-27. Haplotype analysis using mirosatellite marker (D5S1725) in family D. Different types are showed with the arrows. Lanes 2, 3, 4 and 5 are heterozygous for the alleles, lane 1 is homozygous.

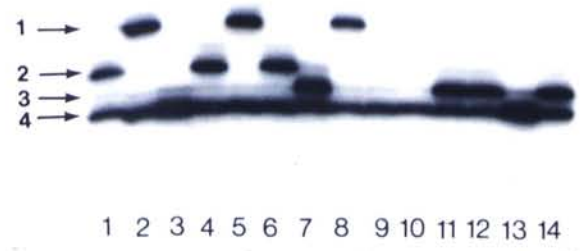

Figure 4-28. An example of haplotype analysis using mirosatellite marker (D5S1462) in family E. Types of alleles are showed with the arrows. Lanes 1, 2, 4→8, 11, 12 and 14 are heterozygous for the alleles detected by these DNA markers. Lanes 3, 9, 10 and 13 are homozygous.

Figure 4-29. An example of haplotype analysis using mirosatellite marker (D5S1470) in family F. Different alleles are showed with the arrows. All subjects are heterozygous for these alleles detected by these DNA markers.

Family A

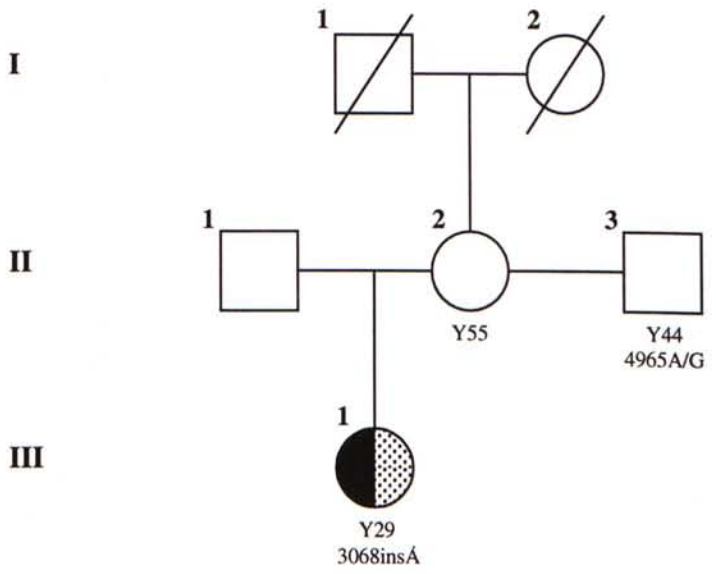

Figure 4-30. The pedigree of family A. Black symbol indicates affected patient with FAP, shadow indicates CHRPE and blank symbols represent unaffected or unknown status at the time of study. Y is the age of the subject was first recruited and examined. II1 is the first husband of II2, II3 is the second husband of II2. 3068insA is a novel mutation and was detected in the FAP patient (III1) in this family. 4965A/G found in II3 is a novel silent mutation.

Family B

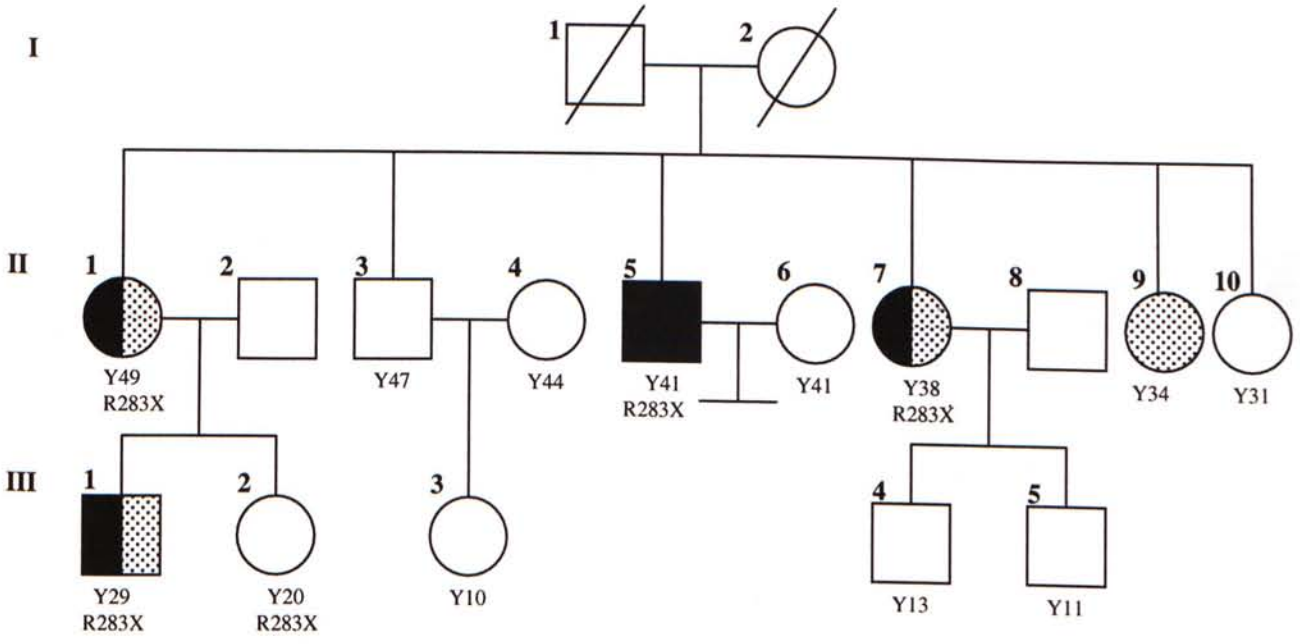

Figure 4-31. The pedigree of family B. Black symbol indicates affected patient with FAP, shadow indicates CHRPE and blank symbols represent the unaffected or unknown status at the time of study. Y is the age of the subject was first recruited and examined. R283X mutation was detected in 4 FAP patients (II1, II5, II7 and III1) and one family member (III2) in this family.

Family C

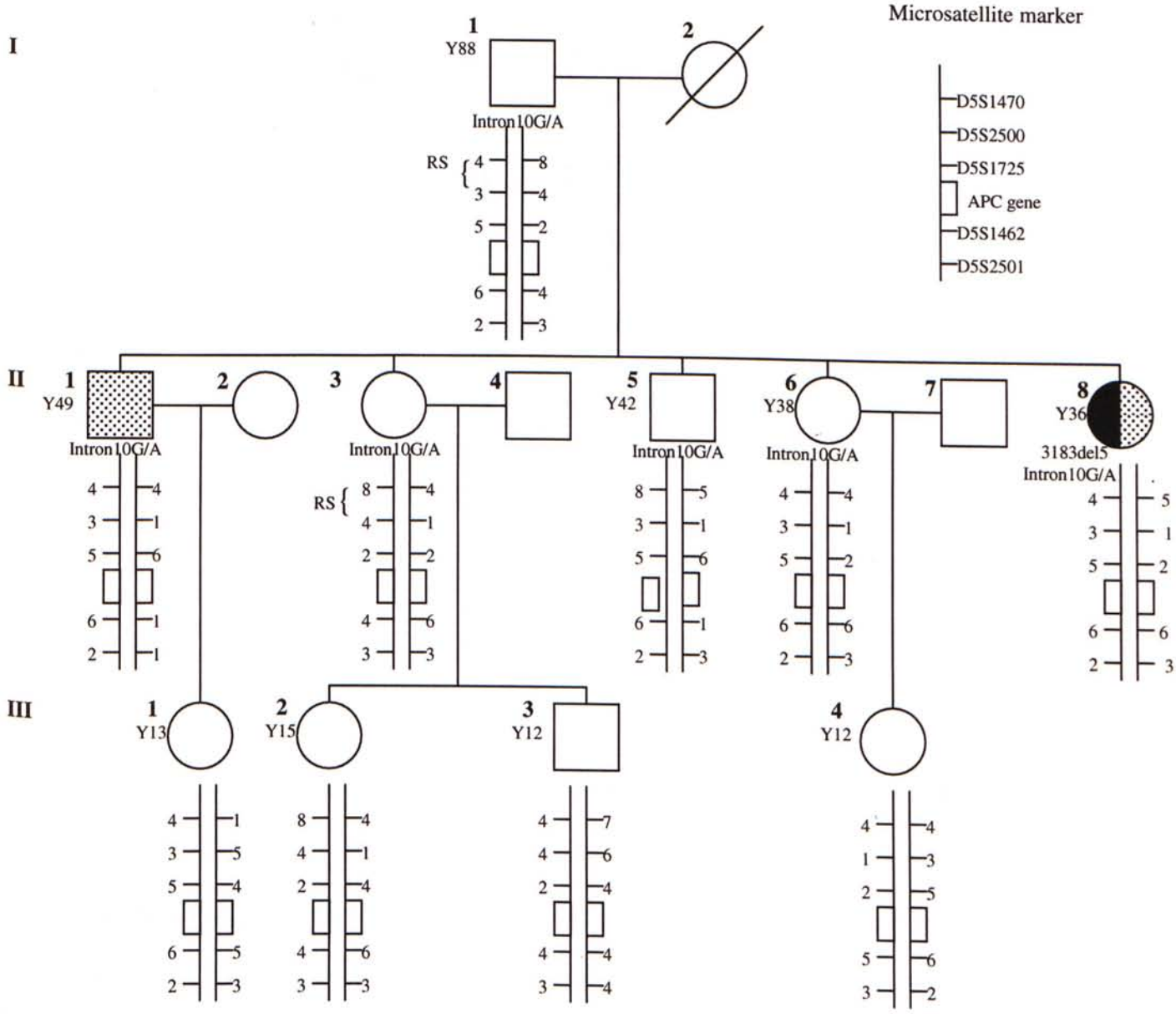

Figure 4-32. Pedigree and haplotype analysis by five microsatellite markers in family C. Black symbol indicates the affected patient with FAP, shadow indicates CHRPE and blank symbols represent unaffected or unknown status at the time of study. Y is the age of the subject was first recruited and examined for this study. 3183del5 mutation was detected in the FAP patient (II8). Intron10G/A is a novel silent mutation and was found in 6 subjects (I1, II1, II3, II5, II6 and II8) in this family. The solid lines below each symbol indicate the two haplotypes obtained for each person. The types of the two alleles are shown for each marker. The recombination sites are indicated by RS.

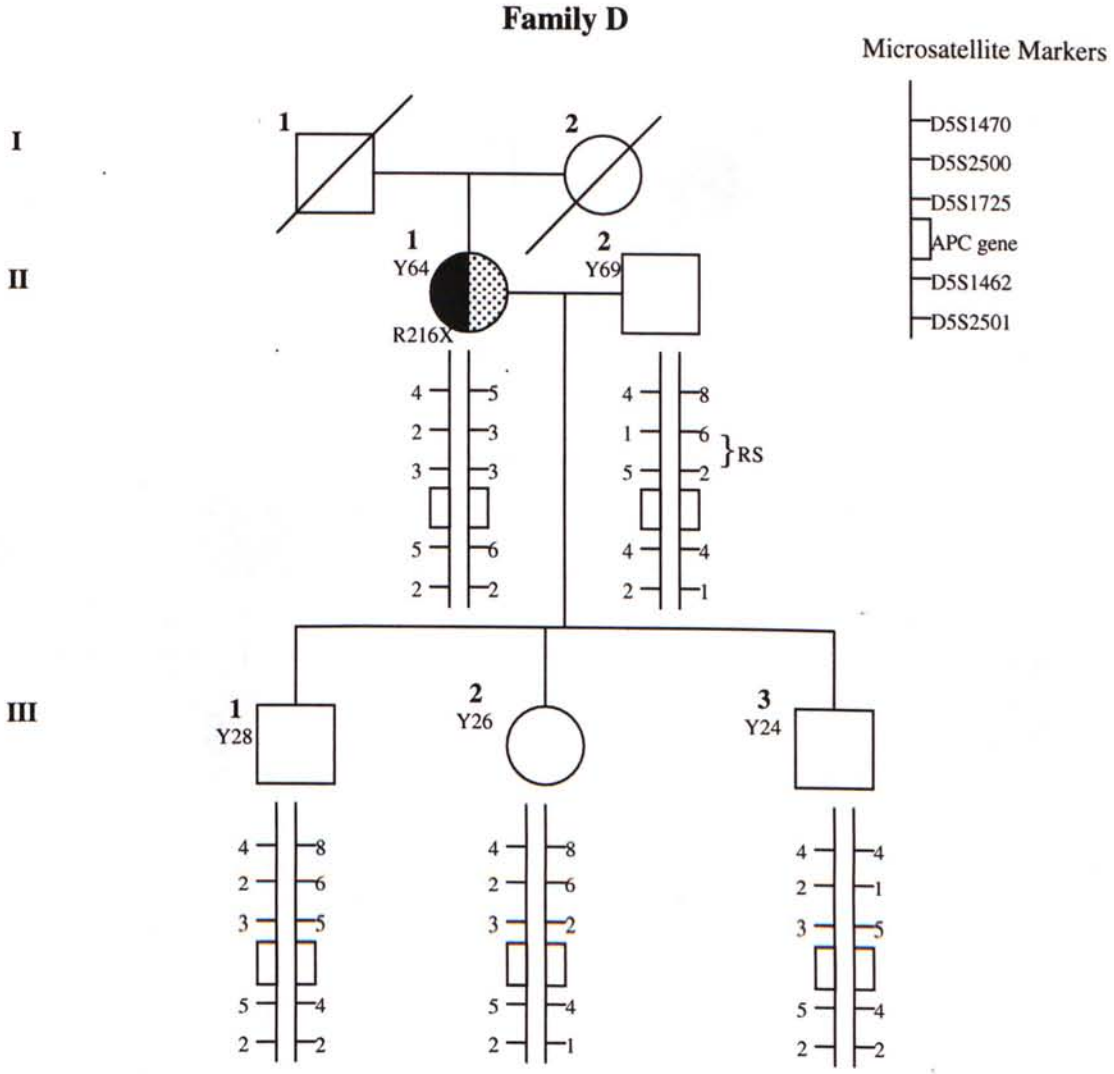

Figure 4-33. Pedigree and haplotype analysis by five microsatellite markers in family D. Black symbol indicates affected patient with FAP, shadow indicates CHRPE and blank symbols are unaffected or have unknown status at the time of study. Y is the age of the subject was first recruited and examined for this study. R216X mutation was detected in the FAP patient (II1). The solid lines below each symbol indicate the two haplotypes obtained for each person. The types of the two alleles are shown for each marker. The recombination sites are indicated by RS.

Family E

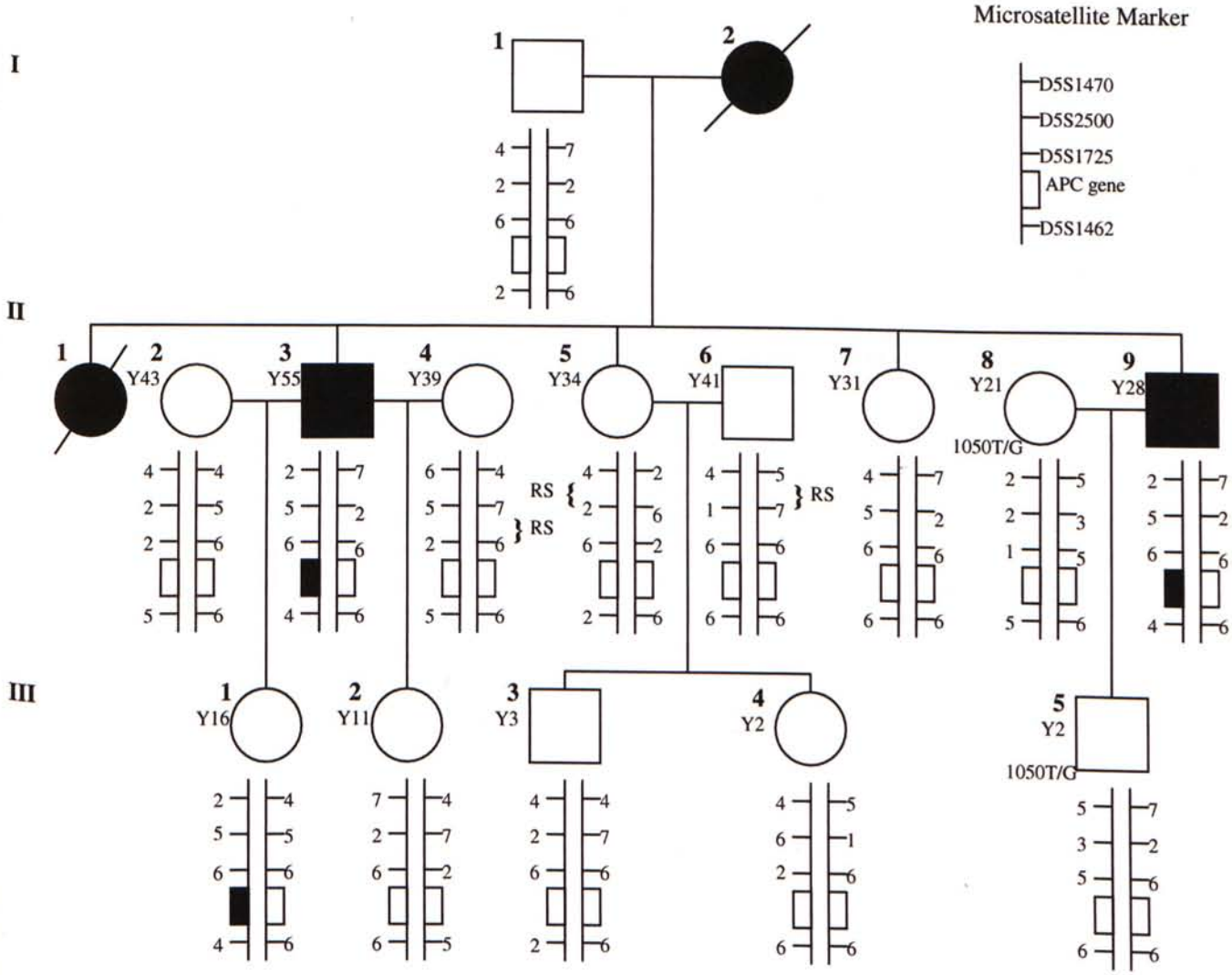

Figure 4-34. Pedigree and haplotype analysis by four microsatellite markers in family E. Black symbols indicate affected patient with FAP and blank symbols are unaffected of unknown status at the time of study. Y is the age of the subject was first recruited and examined for this study. II2 is the first wife of II3 and II4 is the second wife of II3. The novel silent mutation 1050T/G was detected in 2 subjects (II8 and III5) in this family. The solid lines below each symbol indicate the two haplotypes obtained for each person. The types of the two alleles are shown for each marker. The recombination sites are indicated by RS. The haplotype associated with the disease is 2-5-6-4, and is presented in the two affected FAP patients (II3 and II9). The offspring III1 has inherited the haplotype associated with the disease from her father.

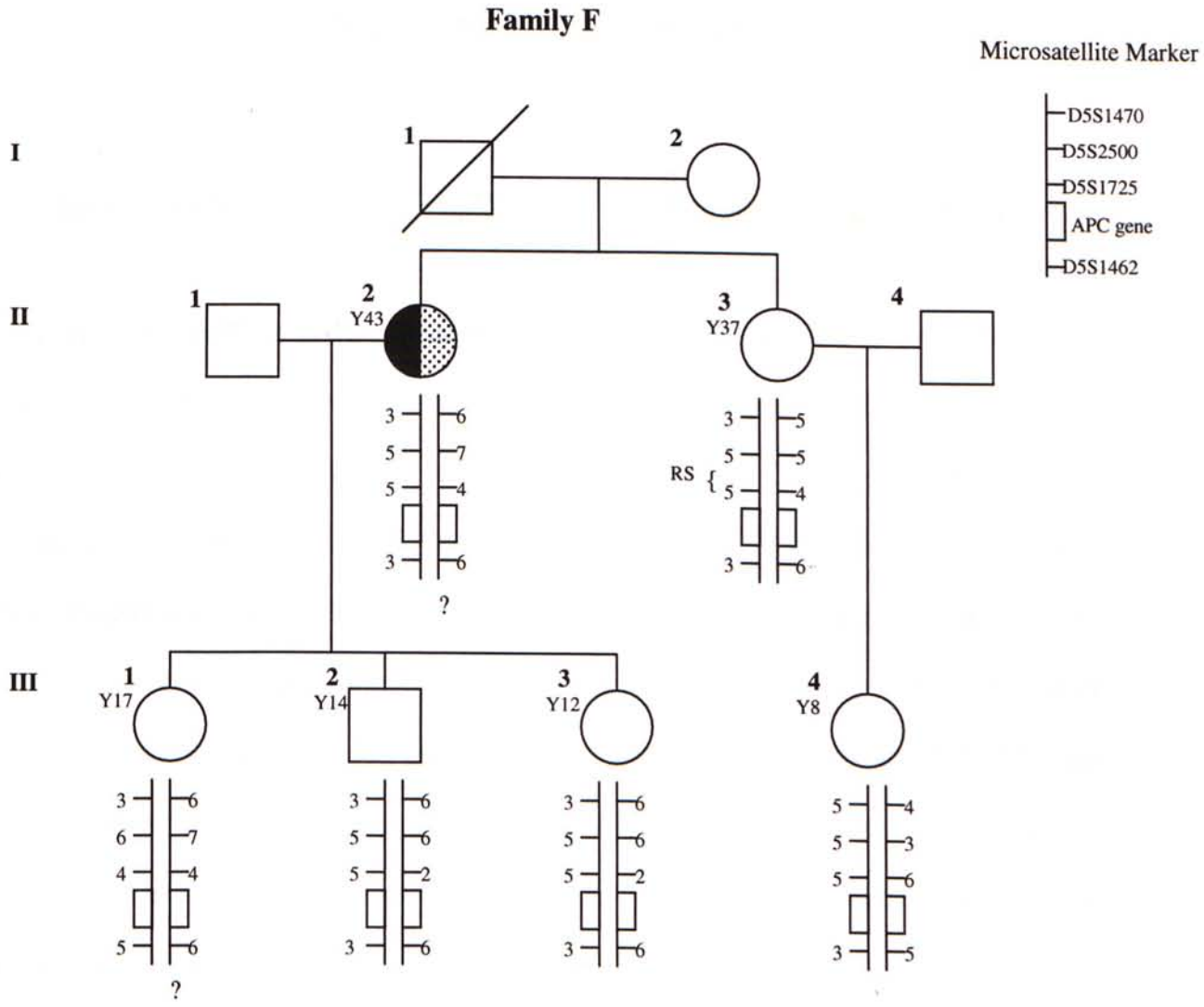

Figure 4-35. Pedigree and haplotype analysis by four microsatellite markers in family F. Black symbol indicate affected patient with FAP, shadow indicates CHRPE and blank symbols are unaffected or have unknown status at the time of study. Y is the age of the subject was first recruited and examined for this study. The solid lines below each symbol indicate the two haplotypes obtained for each person. The types of the two alleles are shown for each marker. The recombination sites are indicated by RS. The question mark indicates the haplotype which is likely to be associated with the disease.

Chapter 5 Discussion

5.1 The predictive value of CHRPE in FAP patients and family members

The incidence of CHRPE in FAP reported in different studies ranges from 66-97% (Berk et al., 1988; Blair et al., 1980; Baker et al., 1988; Burn et al., 1991; Polkinghorne et al., 1990; Romania et al., 1989; Chapman et al., 1989). In our study, the incidence of CHRPE in FAP was 77.8%. The explanation for these variations of incidence is not clear. It is possible that different ophthalmologists may vary in their criteria for recording retinal abnormalities, especially for small lesions, or that patient populations may also differ. In this study, the criteria of CHRPE positive was three or more lesions in one or both eyes, because small numbers of CHRPE may occur in normal subjects (Burn et al., 1991). This criteria is thought to be specific and sensitive for FAP (Lewis et al., 1984; Romania et al., 1989). However, ophthalmologists may not detect all lesions because Traboulsi et al. (1987) identified 70 lesions of CHRPE at autopsy in the retinae of a FAP patient with only 24 CHRPE lesions previously described with fundoscopy. In our study, 4 family members (the ages were 2-3 year old) did not consent for ophthamoscopy, but it was possible to detect CHRPE lesions in them. CHRPE is a congenital condition, it can be observed at any time after birth. Romania et al. (1989) urged that all family members at risk of inheriting FAP should be examined by ophthamoscopy at the earliest age possible. The sensitivity and specificity of CHRPE in FAP were 0.78 and 0.95 in this study. Those figures are close to the reports in the Caucasian and Japanese populations, 0.78 and 0.95 as reported by Traboulsi et al (1987), or 0.862 and 0.997 as reported by Shozo et al (1990). The incidence of CHRPE in possible carriers is close to the calculated value of the expression of the polyposis. A

retinal examination positive for the characteristic RPE lesions signifies a high risk for FAP, whereas a negative retinal examination is uninformative. Althuough CHRPE positive may not always indicate an FAP carrier status and negative may not eliminate an FAP carrier status, but ophthalmologic examination is a direct, noninvasive and inexpensive test, and CHRPE maintains its role in FAP screening, especially in families with known APC mutations. Our results are consistent with a previous study on 11 Chinese FAP patients who all had CHRPE lesions (Lam et al. 1998). CHRPE is a valuable early predictive marker of FAP, and in addition to mutation analysis and linkage study of the APC gene, the possibility for presymptomatic diagnosis of FAP is almost 100 percent. Our results also indicate that establishment of a FAP registry in Hong Kong would be highly desirable in which occular screening and genetic counsel can be provided to FAP patients and their family members.

5.2 The laboratory techniques in this study

5.2.1 PCR optimization

PCR is the basis for the preparation of a target DNA fragment for the detection of base changes in many different methods and it is widely use in genetic studies. However, a successful PCR portocol depends on stringent reaction conditions, so the procedure of PCR optimization is required. Magnesium concentration and annealing temperature were the major factors during PCR optimization in this study. Magnesium ion influences enzyme activity, increases melting temperature (Tm) of double strand DNA and forms soluble complexes with dNTP, which is essential for dNTP incorporation. Higher magnesium concentration could increase nonspecific PCR products that display nonspecific bands on the agrose gel. The magnesium concentration

may be varied from approximately between 0.5mM to 5mM to obtain the optimum. The annealing temperature is the temperature at which the primer is able to bind to the target DNA but are unable to bind to DNA that does not match the target DNA exactly. The annealing temperature is one of the most important factors in PCR optimization. In many situations, exclusive amplification of the desired product is achieved after modifying the annealing temperature. The range of annealing temperature is between 50°C to 65°C (Philip et al. 1992). In our study, twenty-two PCR protocols have been established and they are summarized in Table 4-5. In addition, in order to increase PCR specificity, contamination must be avoided. Some general rules for PCR set-up should be followed for preventing PCR contamination, such as: using personal reagent sets and pipets, wearing gloves and changing them frequently, and using disposable materials. For every PCR, negative water control should be needed to check PCR reagent for contaminations.

5.2.2 Single strand conformation polymorphism (SSCP)

Single strand conformation polymorphism (SSCP) is used widely for detecting both mutations and polymorphisms for heterzygous or homozygous. The sensitivity of SSCP for detecting point mutations can be improved by changing a variety of SSCP conditions. Factors that affect the sensitivity of SSCP include the length of the PCR fragment, temperature of the running gel, the method of PCR denaturation, composition of gel and electrophoretic conditions (Hayashi, 1991). The electrophoresis temperature is an important factor in detecting single strand comformation. At lower temperatures, electrophoretic mobility was retarded. The single strand DNA mobility was also dependent on temperature, so it was necessary to ensure that temperature gradients within the same gel were minimized by sufficient cooling for the heat generated. Gel

composition is the another important factor for effecting the sensitivity of SSCP. The extent of cross-linking is expressed by the ratio of N,N′-methylbisacrylamide to total acrylamide concentrations, the ratio affected both DNA as well as indicator dye migration (Dean et al. 1991). Glycerol content of the gel affected the relative mobilities of single strand DNA, because of their denaturant properties on the secondary conformation of macromolecules (Orita et al. 1989). In this study, every SSCP was performed under two conditions to increase sensitivity: 6% acrylamide gel containing 5% glycerol with acrylamide:bisacrylamide ratio of 49:1 at room temperature and 6% acrylamide gel containing 10% glycerol with acrylamide: bisacrylamide ratio of 37:1 at 4°C. Eleven SSCP positive conformers were detected using this method, 8 of them were found in both conditions, 3 were just found in 10% glycerol at 4°C. The SSCP condition, 6% acrylamide gel containing 10% glycerol with acrylamide: bisacrylamide ratio of 37:1 at 4°C, ensure high sensitivity. It identified that the conformation of single strand DNA which can change by different gel temperature used during electrophoresis (Hayashi, 1991). The reported sensitivity of detecting mutations with SSCP was about 70% (Hayashi, 1992). In this study, 2 conditions were used to increase the sensitivity of SSCP over 90%.

5.2.3 Direct DNA sequencing

Direct DNA sequencing is taken to identify the base difference for the SSCP positive conformers. In this study, 4 nonsense mutations, 4 polymorphisms and 3 slient mutations were detected using direct DNA sequencing. It is an effective method for detecting all kinds of mutations and has been widely used in many laboratories. But the method is laborious and radioisotopes have to be used. Automated sequencing will overcome the disadvantage, but it needs time to set up the programe in routine practice.

5.3 Novel mutations in the APC gene

The novel mutation 3068insA that creates a stop codon 17 nucleotides downstream is a one base pair (A) insertion at codon 1023 in exon 15 of the APC gene. We found the mutation in a 29-year-old woman (AIII1) in family A. She had been clinically diagnosed with FAP and prophylactic colectomy was performed on her. She is heterozygous for the mutation, and fifteen CHRPE lesions were observed in both eyes. It is difficult to study the mutation due to small family members in this family. However, it is necessary to analyze the novel mutation in a large number of Chinese FAP patients in future.

5.4 Reported mutations in the APC gene

5.4.1 3183del5.

The mutation that is a 5 base pair deletion of ACAAA at codon 1061 is a hot spot in the APC gene and has been reported by several groups including Japanese and Caucasians. Michiko et al (1995) reviewed more than 100 germline mutations of the APC gene in 400 FAP families and found 3183del5 mutaion in 14 FAP families. This mutation produced a frameshift and created a stop codon (AAG) within 2 nucleotides. The mutation identifies the rule that deletions in the APC gene often occur at positions containing repeated sequences within the coding sequence, and cause frameshifts, which result in stop codons further downstream. We detected the mutation in a 36-year-old woman with FAP who had undergone colectomy several years previously. She is heterozygous for the mutation, and 18 CHRPE lesions were found in both eyes.

5.4.2 R216X and R283X

The mutation R216X was first reported in 2 of the 70 Japanese FAP patients (Miyaki et al. 1994), and R283X mutation was detected in one of the 100 FAP patients in French (Olschwang et al. 1993). The two mutations are C to T substitutions which change arginine (CGA) to a stop codon (TGA). APC germline mutations are more than 80% base substitutions from cytosine to other nucleotides. It can be explained by the theory that a large number of mutations from C and G occurs as a result of transient misalignment of the bases at the replication fork (Cooper et al. 1990). It can also be explained by the fact that about 98% of mutations in the APC gene result in stop codons TGA, TAG and TAA. R216X was detected in a 64-year-old woman (DII1) who was an FAP index patient and had colectomy. She was heterozygous for the mutation. R283X was found in 5 study subjects in the family B, including 4 FAP affected patients and 1 family member. They are all heterozygous for the mutation.

5.5 Silent mutations and Polymorphisms in the APC gene

5.5.1 Novel silent mutations

Ser 1050 Ser in exon 9

The novel silent mutation found in exon 9 of the APC gene was a T→G base change at nucleotide position 1050, which does not alter the amino acid (serine). We detected this silent mutation in a family member (EIII5) and a normal control (EII8). This silent mutation may not be linked to FAP or CHRPE (Table 4-3), but it is necessary to screen for this silent mutation in a large number of FAP affected patients and normal controls.

G→A Transition at exon 10 and intron 10 boundary

The novel silent mutation is a transition 5bp downstream from the exon 10-intron 10 boundary. It is unlikely to be a splice site mutation because the probability of a mutation in the (-6) position to give a 3' splice site mutation is only 12% (Krawczak et al. 1992). The novel silent mutation was found in 6 study subjects including 1 FAP index patient and 5 family members in family C. It is also necessary to screen the silent mutation in a large number of FAP affected patients and normal controls for study the relationship between the silent mutation and FAP.

Thr 4965 Thr in exon 15

The novel silent mutation was an A→G base change at nucleotide position 4965, that does not alter the amino acid (threonine). Because the silent mutation was just found in a normal control, it is necessary to screen more FAP index patients and normal controls to determine the features of this silent mutation.

5.5.2 Polymorphisms

1458C/T in exon 11

1458C/T is a common polymorphism in the APC gene. Nagase et al (1992) tested 150 individuals and found frequency of the polymorphism was 0.43/0.57 (with or without an Rsal site). We detected the polymorphism in 31 of 55 study subjects, 22 of the 31 were heterozygous and 9 were homozygous. Frequency of the rare allele (T) was 0.35 in FAP patients, 0.29 in family members and 0.75 in normal controls. The polymorphism is not specific to FAP patients, in agreement with the previous report by Nagase et al. in 1992.

1635A/G, 4479G/A and 5043A/G in exon 13 and 15

Frequencies of these 3 polymorphisms were reported to be 0.34/0.66 in 1635A/G, 0.35/0.65 in 4479G/A and 0.64/0.36 in 5043A/G with or without cutting site (Miyoshi et al. 1992, Powell et al. 1992 and Nagase et al. 1992). These 3 polymorphisms are very common in the APC gene. In this study, it is interesting to find that the three polymorphisms occurred together in 8 study subjects, including 1 FAP index patients, 3 family members in 3 different families and 4 normal controls. Seven of the 8 study subjects were heterozygous and one was homozygous for the three polymorphisms. Frequencies of the rare alleles for the 3 polymorphisms were 0.05 in FAP affected patients, 0.045 in family members and 0.31 in normal controls. Frequencies of the 3 polymorphisms in this study were different from other reports may be due to small individuals tested. However, we could not find relationships between the 3 polymorphisms and FAP or CHRPE (Table 4-3) that agree with the reports of other groups (Miyoshi et al. 1992, Nagase et al. 1992 and Powell et al. 1992).

5.6 The relationship between the APC gene mutation and CHRPE

The correlation between location of mutations in the APC gene and CHRPE in FAP patients was reported (Olschwang et al. 1993, Wallis et al. 1994). Retinal lesions are almost absent if the mutation occurs before exon 9, but are consistantly present if occurs after this exon. However, our study showed different results. Table4-2 summaries FAP, CHRPE and APC mutations in all 10 FAP patients in this study. The mutation R216X was detected in exon 6 of the APC gene in an FAP affected patient (DII1), and 5 CHRPE lesions were observed in her both eyes. The mutation R283X was found in exon 8 of the APC gene in 5 study subjects including 4 FAP

affected patients (BII1, BII5, BII7 and BIII1) and 1 family member (BIII2). CHRPE lesions were observed in 3 of the 5 study subjects. Our study indicated CHRPE lesions are present if the mutation occurs before exon 9 of the APC gene. Mutations 3068insA and 3183del5 were detected in exon 15 of the APC gene in 2 FAP index patients (AIII1 and CII8) respectively and both of them showed more than 15 CHRPE lesions in both eyes. CHRPE lesions perhaps are serious if the mutations occur at codons 1023 and 1061 of the APC gene. But further analysis of a large number of patients is required to determine whether the position of the APC gene mutation is correlated with CHRPE. We detected APC mutations in only 7 of the 10 FAP patients (70%). One of the reasons may be SSCP analysis is not sensitive enough to detect all mutations. Moreover, we finished screening the APC gene from exon 6 to 15 (codon 216-2843), but exon 1-5 were not tested. However, about 9% of the mutations occurred in exon 1-6 of the APC gene (Joyce et al. 1995). The author reviewed 174 germline mutations in the APC gene, only 16 mutations in exon 1 to 6. However, we detected one APC mutation R216X in exon 6 in the 10 FAP patients, it is necessary to screen exon 1-5 of the APC gene in Chinese FAP patients.

5.7 Haplotype analysis

Haplotype analysis is a rapid and efficient method for analyzing the genotype with hereditary disease and presymptomatic diagnosis of FAP. In this study, we attempted haplotype analysis using 5 different closely linked and polymorphic microsatellite markers flanking the APC gene in 35 individuals consisting of 4 FAP families. The haplotype associated with disease was detected in Family E (Figure 4-34). However, the alleles associated with disease could not be determined in the other 3 FAP families due to the small number of family members studied. We

should not ignore the rare possibility of recombination. A combined use of the polymorphisms near or just in the APC gene, and making a haplotype of the gene for analysis, will raise the informativity and reliability further. The recombinant events involved D5S1470 and D5S2500 markers, therefore we were able to clarify that these two markers are farther away from the APC gene than D5S1725 and D5S1462. Genetic mapping gives only a crude estimate of the size of the critical region, beacause genetic distances between markers are usually known with much less certainty than the map order. Up to now, distances between the APC gene and these markers have not been reported and there is no publications about using these 5 microsatellite markers (D5S1470, D5S2500, D5S1725, D5S1462, D5S2501) to perform haplotype analysis in FAP families. In our study, these markers showed high heterozygosity with high allelic frequency (Table 4-9) and multiple bands (Figure 4-26 to 4-29). Whether new polymorphisms discovered in the Chinese FAP patients in this study are suitable for use as polymorphic markers for haplotype remain to be investigated. Although the use of haplotype segregation analysis is widespread for presymptomatic diagnosis, its informativeness is still limited in some cases, such as: key family members being unavailable, linkage markers not informative in the family, or a sporadic case with few family members.

Chapter 6 Conclusion

1. CHRPE is an early clinical marker with high sensitivity, specificity and predictive value for FAP. Opthalmologic examination is a direct, noninvasive and inexpensive test in FAP screening.
2. The novel and all 3 previously reported mutations found in this study are nonsense mutations and segregated with CHRPE.
3. Our study indicated that the site of an APC gene mutation influences CHRPE expression but is not the only factor responsible for the presence and number of retinal lesions in FAP patients.
4. Indirect genotyping of FAP with highly polymorphic microsatellite markers flanking the APC gene maybe is a rapid, efficient, and highly reliable method for presymptomatic diagnosis of FAP.

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